

1991

## Project Reports

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*Utah State University*

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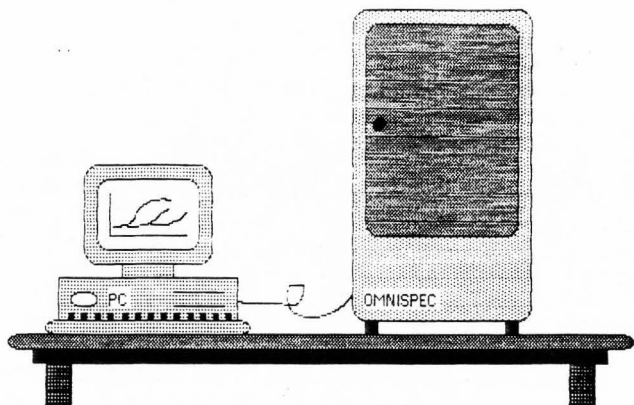
Prediction and determination of the efficacy of nisin in dairy foods

Studies on growth and survival of *Bifidobacterium* species in milk

Characterization of milk proteolysis by lactococcal starter culture strains using amino acid analysis

# Curd Formation/Cheese Technology

## The OMNISPEC Biomonitor



The "OMNISPEC Biomonitor" is an automated instrument with numerous applications in dairy, food, medical, and environmental laboratories. The instrument consists of an incubator containing robotics that automatically and continuously rotate Microtiter™ plates over a reflectance color meter detector module. The detector uses the entire visual spectrum thus allowing incorporation of any of the traditional dyes used in microbiological research and quality control. Changes in color are recorded and used to detect previously selected endpoints. The endpoints allow calculation of the original bioburdens in the samples. Modification of media and dyes allows detection of different types of bioburden. The instrument may be coupled with an IBM-compatible microcomputer/controller that prints out all results automatically.

The above unit is referred to as the "analyzer robot". Sample preparation for inclusion in this unit would require manual dilutions, etc. When combined with a Zymark "sampler robot", the unit would conduct all of the steps associated with preparing Stomached or blended homogenates for introduction into the incubator. For example, the "sampler robot" could transfer a test tube from an ice bath to a pasteurizer bath with 30 minute  $\pm$  1 sec precision for a laboratory pasteurized count test! It could then transfer the heated and cooled sampled, add reagents, fill the plates and transfer

them to the "analyzer robot". The combination of these two units would make larger laboratories significantly more productive.

### **Test Capabilities:**

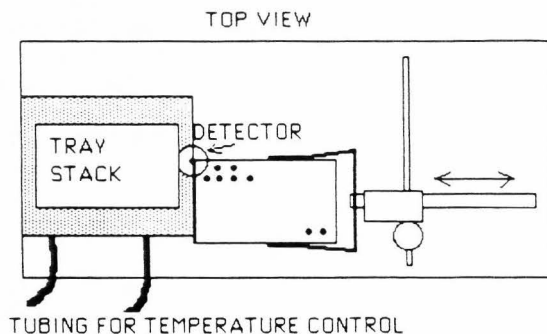
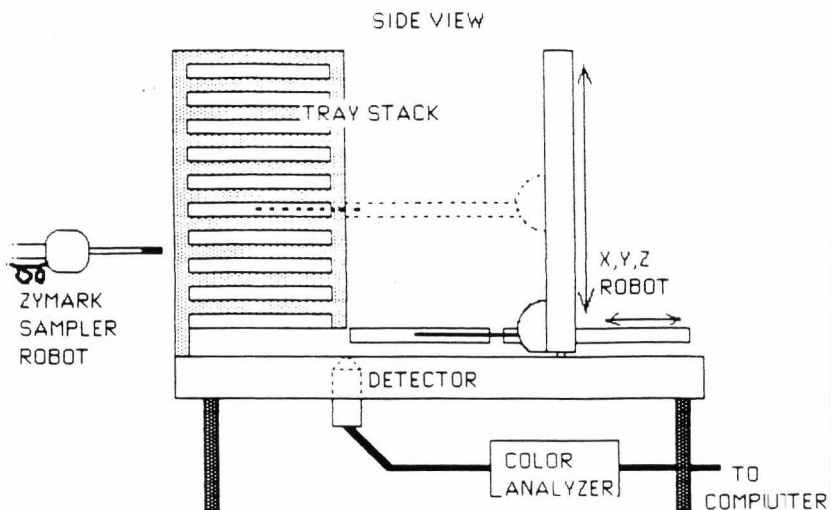
- Rapid bioburden assays (equivalent to most probable number, aerobic plate count, etc. techniques) to less than one colony forming unit (cfu)/ mL in 3 to 33 hours, depending upon the microbial load.
- Antibiotics assays in 0.5 to 2 hours to detect nonspecific inhibitors of lactic cultures down to 0.0005 International Units (IU)/ mL of penicillin equivalents. The instrument can also provide greater precision and quantitation than other with tests, such as the *Bacillus sterothermophilus* methods.
- Abnormal milk detection available immediately upon first reading of samples in incubator. The pH and chloride ions can be measured.
- Coliforms, laboratory pasteurization counts (spore counts), psychrotrophs (gram negatives), shelf life predictions, yeast counts, staphylococci counts, culture activity tests, etc., can be determined more rapidly than with traditional methods. Only media and procedures need modification to change tests. The computer can be programmed to detect any combination of samples within a Microtiter™ plate.
- Agglutination of lactic cultures and culture reduction of a medium can be measured.
- Proteolytic and lipolytic enzymes can be quantitated.
- Opaque, turbid, and clear solutions can be measured using all three parameters of an L\*a\*b\* color system.
- Color quality of foods can be monitored.

### **Advantages:**

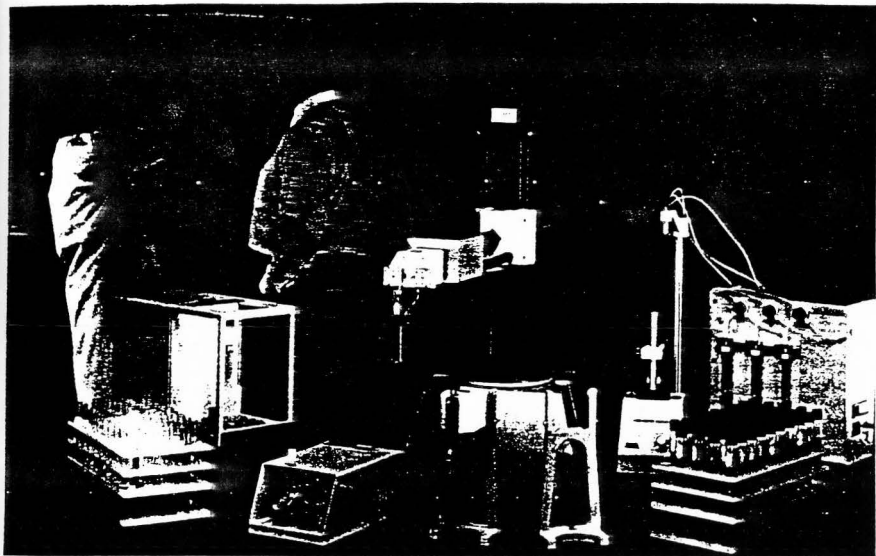
- Versatility allows from one to 1920 samples per day in a single incubator. With two incubators 3840 samples can be simultaneously monitored.
- Data generated relates better to important parameters like shelf life or sterility or post pasteurization contamination than traditional plate counts.
- Results are available much faster than with traditional plate counts.
- The same dyes that have been traditionally used in microbiology can be employed; i.e. resazurin, brom cresol purple, brom thymol blue, TTC, CVT, litmus, etc.
- There is no temperature or electrical stabilization time requirements.
- Tests can be interrupted or removed from incubator without causing problems with the endpoints.

- Low cost disposables are below 2¢ each!
- Samples and plates can be prepared manually using traditional Microtiter™ pipets and diluters. Sample preparation, plate preparation, and plate introduction into incubator can be automated through incorporation of a Zymate II Robot (Zymark Corp. Hopkinton, MA 01748).
- Assays can be mixed within one plate since detector/computer can apply different endpoint requirements to each well.
- Fewer methods require solid media.
- Preincubation of homogenized (Stomached) samples allows detection of less than 1 cfu/ mL.
- Minimal labor requirements.
- Totally automated incubation, data collection, and printouts.

G.H. Richardson, 27 Dec 1986



ANALYZER ROBOT SIDE AND TOP VIEWS,  
OMNISPEC, G.H. RICHARDSON, 6 JUNE 1987



The Zymate System performs automated procedures for a broad range of applications — from routine quality control tests to process control procedures to specialized research techniques. Zymate Applications fall into one of two categories — custom or standard.

A custom application is a procedure which is automated to duplicate your existing manual method as nearly as possible. Your Zymate representative will review your detailed procedure and recommend a Zymate System configuration to meet your application requirements. A Zymark Systems Engineer is assigned to each customer order to plan the application in detail and insure that the required modules and accessories are provided. Some preliminary applications programming will be provided, and you will receive training to complete the implementation of your procedure.

The following list illustrates the wide variety of Zymate applications in a diverse number of industries.

#### **General Sample Preparation**

Automated Weighing  
Automated Extractions  
Chromatography (HPLC and GC)

#### **Sample Introduction**

Spectrophotometry  
Nuclear Magnetic Resonance  
Differential Scanning Calorimetry  
Tensile (Instron) Testing

#### **Hazardous Work Environments**

Radioactive, toxic and carcinogenic chemicals  
Cold and low humidity conditions  
Ultrasonic Exposure

#### **Pharmaceutical**

Dissolution Testing  
Content Uniformity  
Tablet Assays  
Karl Fischer Titrations  
Pyrogen Testing (LAL Assay)

#### **Biochemistry**

Total Protein Assays  
Enzyme Kinetics  
Receptor Binding Assays  
Genetic Engineering  
Immunoassays (ELISA, RIA, EIA)

#### **Chemical**

Elemental Analysis  
Automated Titrations  
Polymer Testing  
Wet Chemistry Procedures  
X-Ray Diffraction Analysis

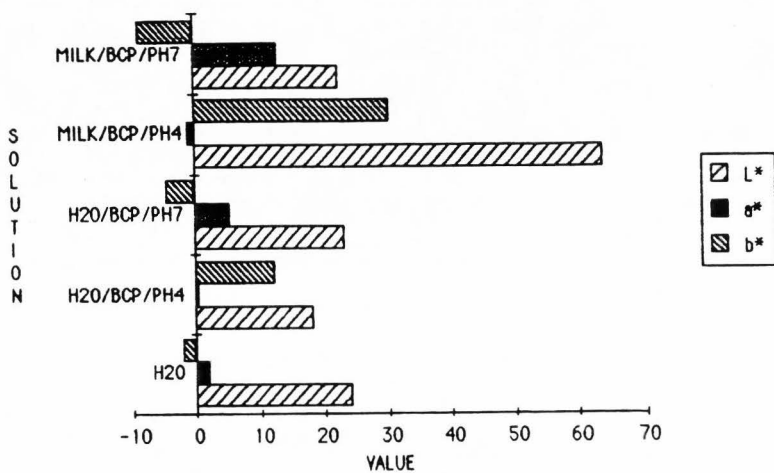
#### **Environmental**

Biological Oxygen Demand  
Total Organic Carbon  
Total or % Solids Assays  
EPA Methods 601 and 602

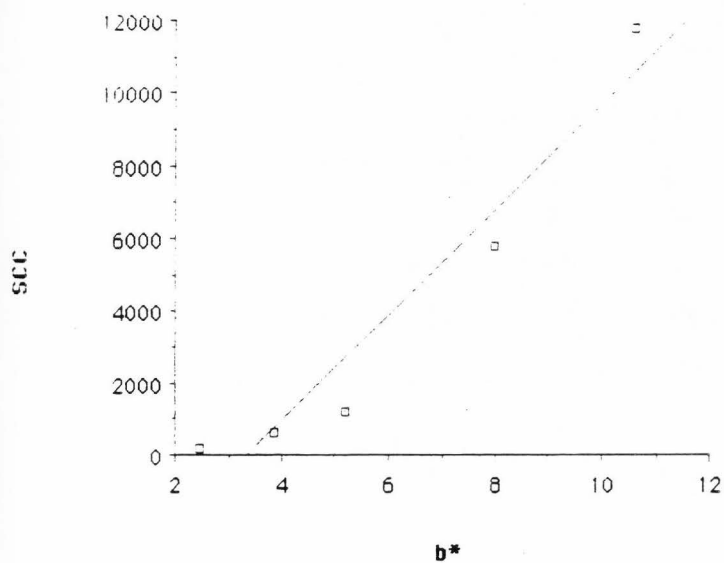
#### **Food and Beverage**

Nutritional Testing  
Dye Analysis  
Fat and Moisture Content  
Protein Analysis

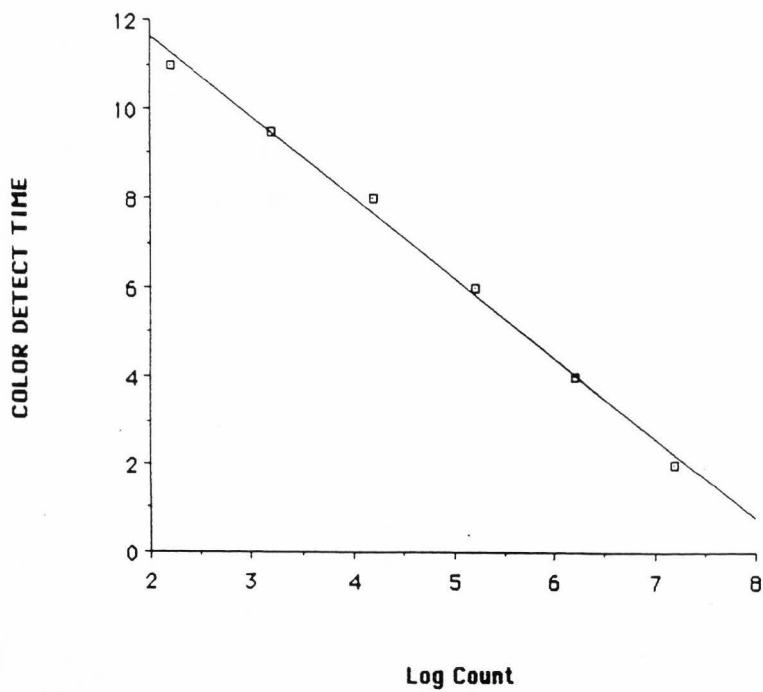




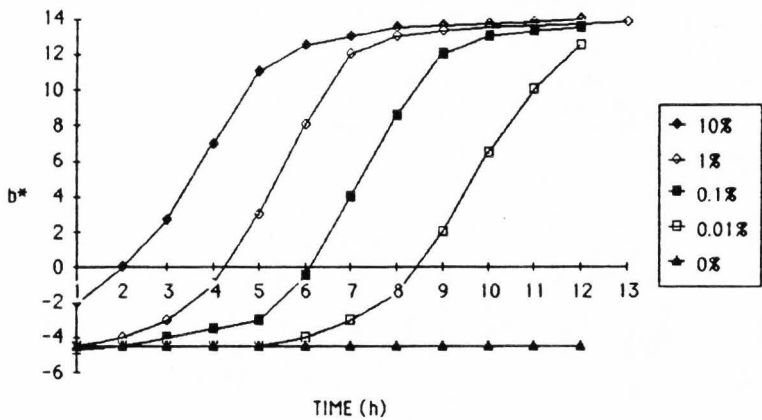
$$y = -4340.134 + 1456.771x \quad R = 0.96$$



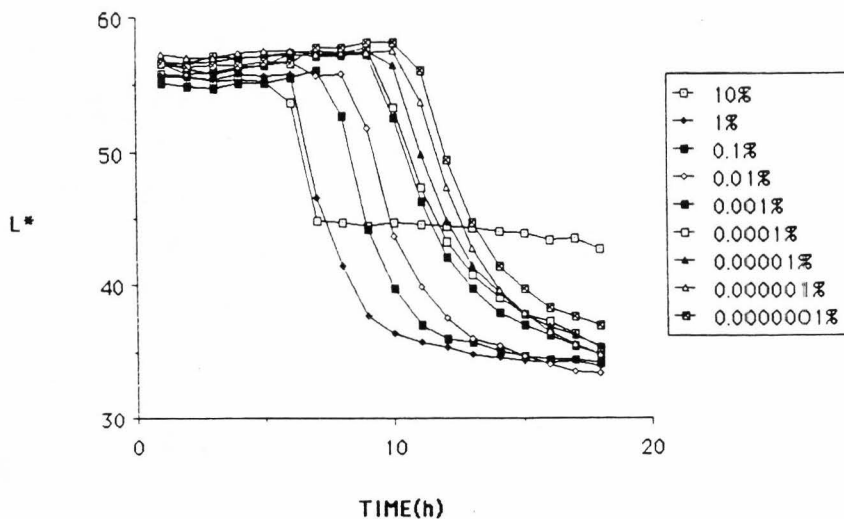
$$y = 15.277 - 1.814x \quad R = 1.00$$



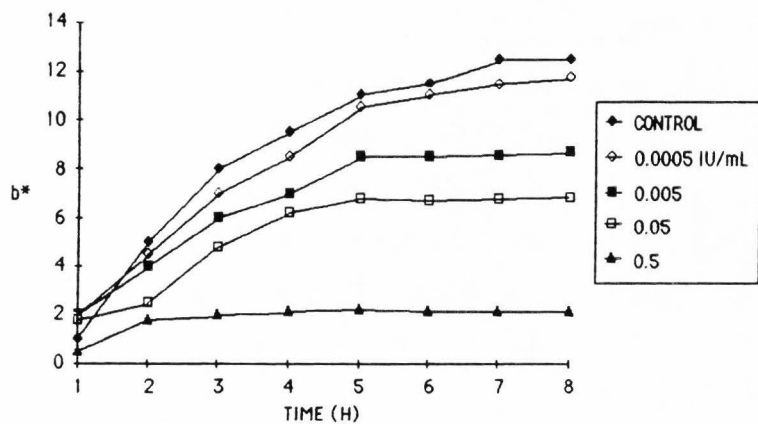
## TOTAL COUNT/BCP



# PLATE COUNT/TTC

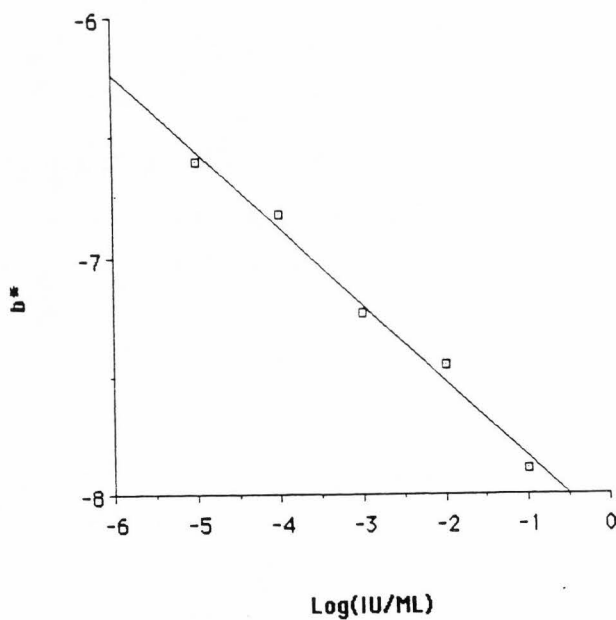


# PENICILLIN/BCP

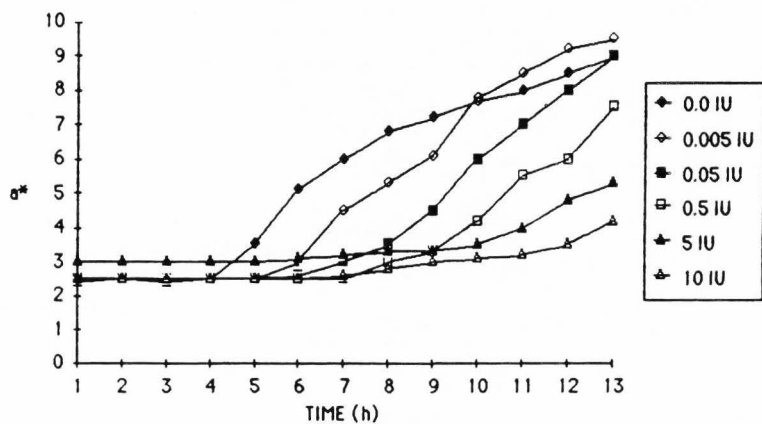


PEN. / 320 + / A-4 / LIT / 175 mth.

$$y = -8.161 - 0.321x \quad R = 0.99$$

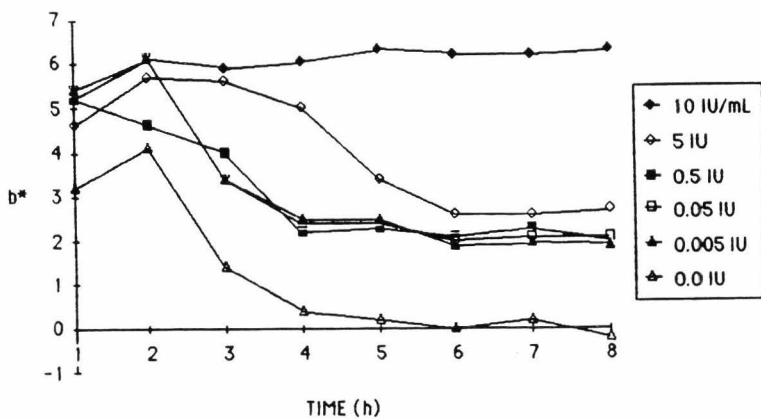


# STREPTOMYCIN/TTC

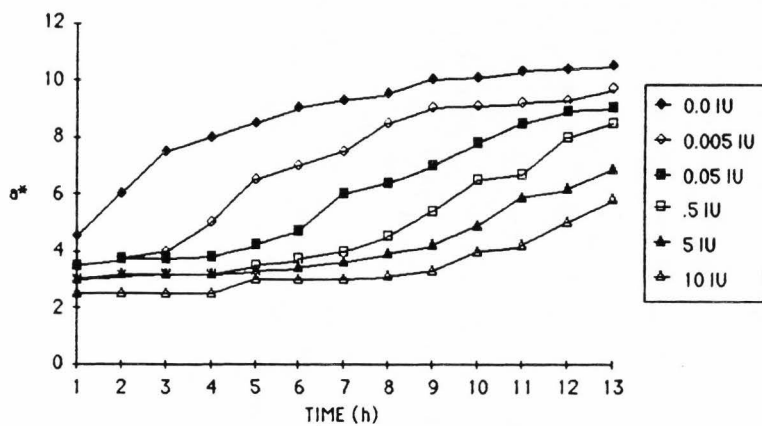


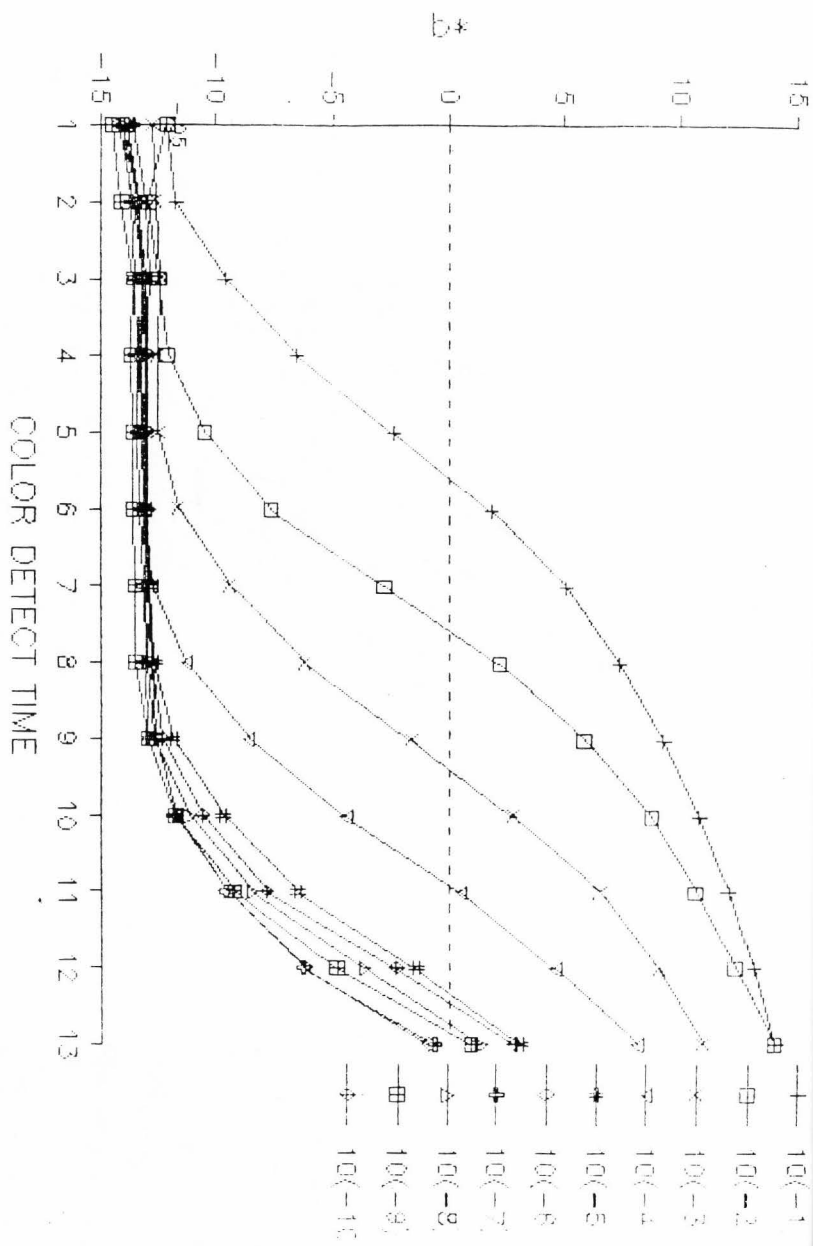


# STREPTOMYCIN/LITMUS

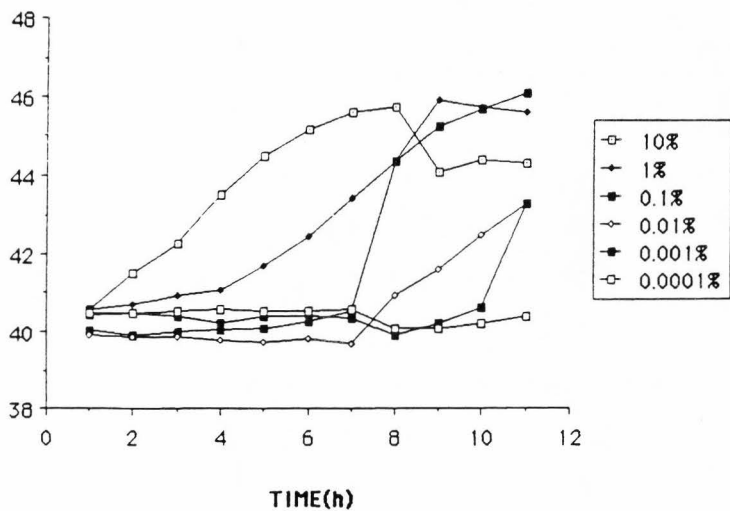


# KANAMYCIN/RES

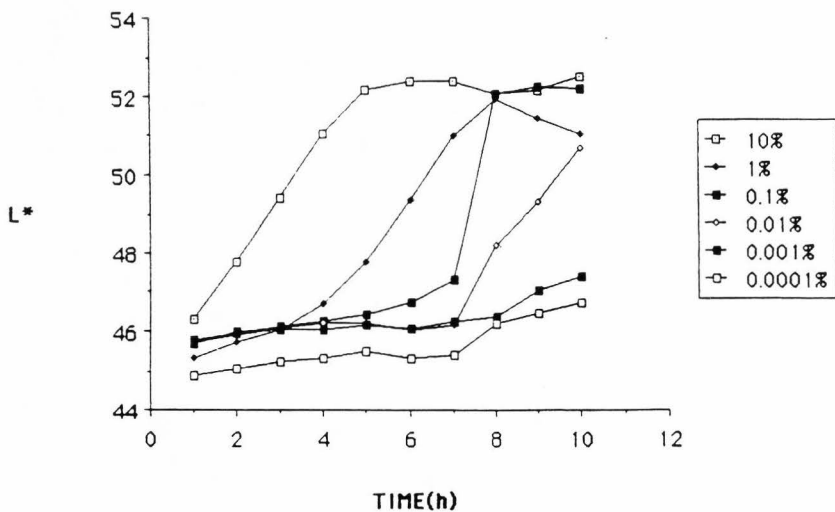




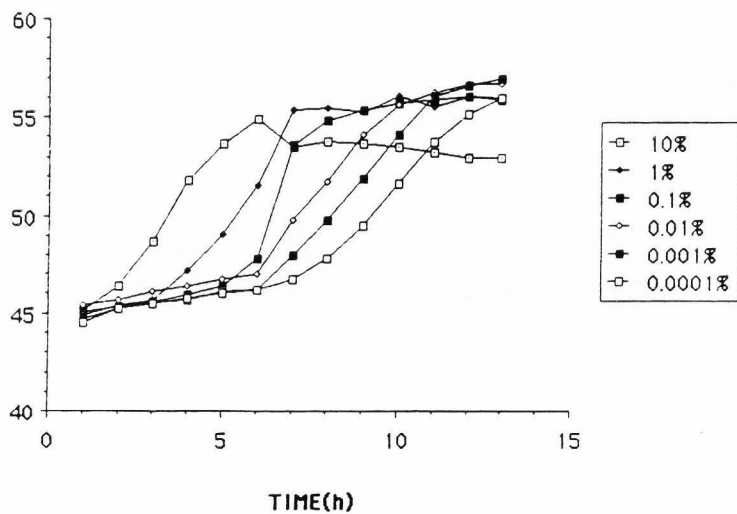
# E. coli COUNT/BCP



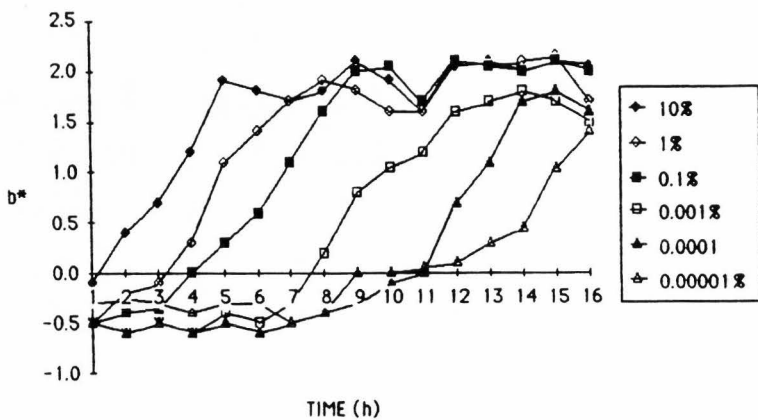
E. coli COUNT/LIT



# E. coli COUNT/RES



## COLIFORMS/BCP

*in Sterile Ice Cream*

**Project Title:** Prediction of cooling rate of Cheddar cheese blocks: Effect of cheese composition and cooling method

**Personnel:** J.A. Torres, Assistant Professor, Food Science and Technology, Oregon State University

F.W. Bodyfelt, Professor, Food Science and Technology, Oregon State University

C. Grazier, Graduate Research Assistant, Food Science and Technology, Oregon State University

R. Simpson, Graduate Research Assistant, Food Science and Technology, Oregon State University

J. Bouzas, Graduate Research Assistant, Food Science and Technology, Oregon State University

**Funding Sources:** Western Dairy Foods Research Center  
Tillamook County Creamery Assoc. (Tillamook, OR)  
Oregon State University Agricultural Experiment Station  
Utah Agricultural Experiment Station

### Objectives:

The goal of this project is to examine the cooling of Cheddar cheese blocks before cheese aging which is believed to be one of the last processing steps requiring tighter control to achieve a more uniform and consistent cheese quality. This would increase consumer confidence in the product and increase Cheddar cheese consumption.

The specific objectives of this research project are:

1. To demonstrate that a more consistent quality can be achieved by controlling the cooling rate of the Cheddar cheese blocks to be aged.
2. To develop a computer program that calculates cooling rate as a function of cooling conditions and Cheddar cheese composition. This program will be made available to Cheddar cheese manufacturers.
3. To identify a starter bacteria to adventitious microorganisms ratio associated with a desirable Cheddar cheese quality as identified by sensory analysis and confirmed by chemical analysis.
4. To use a combined heat transfer and microbial activity model to determine the cooling rate that gives after cooling a desirable starter bacteria to adventitious microorganisms ratio.



**Project Title:** Cheddar cheese blocks: Effect of cheese composition and cooling methods

**Personnel:** J. Antonio Torres, Principal Investigator, Dept. of Food Sci. & Technology, Oregon State University

Floyd W. Bodyfelt, Principal Investigator, Dept. of Food Sci. & Technology, Oregon State University

Conly L. Hansen, Principal Investigator, Dept. of Nutrition and Food Sciences, Utah State University

Jorge Bouzas, Ph.D. Graduate Student, Dept. of Food Sci. & Technology, OSU

Connie Grazier, M.S. Graduate Student, Dept. of Food Sci. & Technology, OSU

Ricardo Simpson, M.S. Graduate Student, Dept. of Food Sci. & Technology, OSU

**Funding Sources:** Western Dairy Foods Research Center  
Tillamook County Creamery Assoc. (Tillamook, OR)

**Objectives:**

1. To develop a computer program that calculates cooling rate of 40 lb and 640 lb blocks as a function of cooling conditions and Cheddar cheese composition.
2. To demonstrate that a more consistent quality can be achieved by controlling the cooling rate of 40 and 640 lb Cheddar cheese blocks.
3. To evaluate the sensory properties of a commercial Cheddar cheese.
4. To identify temperature conditions leading to sensory properties similar to commercial Cheddar and confirmed by chemical and microbial analysis.
5. To use a combined heat transfer and microbial activity model to determine the cooling rate and aging room temperature that result in the sensory quality identified in (4) in a uniform manner.
6. To quantify the effect of heterogeneous temperature distributions and microbial activity on the chemical and sensory characteristics of 640 lb cheese blocks.

7. To use a mathematical optimization method to determine cooling conditions that reduce sensory differences between the various locations in 640 lb cheese blocks.

## Results:

The experimental procedures used in this research were developed primarily to accommodate the wide range of temperature effects to be covered (5-35°C) and the large experimental error associated with sensory analysis. Samples were drawn from commercial production after the pressing step in the process used at Tillamook County Creamery Assoc. (Tillamook, OR). Blocks were cut and vacuum wrapped under sanitary conditions. Samples were small enough to reach storage temperature within 1-2 hours. The experimental design includes two batches with two replicates stored at 5, 15, 25 and 35°C with results of the effect on the sensory and microbial parameters reported last year. An additional four batches with one replicate have been tested this second year. Sensory and microbial analysis of these samples have been completed. Chemical analysis is underway and will be completed this summer. Chemical analysis is underway and will be completed this summer. Engineering and modeling efforts will be completed by December 1990.

## Impact of Research:

American is the major type of cheese produced in the United States—the vast majority of this is Cheddar cheese. We believe that Cheddar cheese sales could increase if there was less variability in product quality. The economical impact of current variability is enormous because much Cheddar cheese has to be marketed at a younger age than is perhaps optimal for best price. The frequency of the sourness/bitterness defect reduces its quality image, reduces its market value and also limits its overall consumer acceptance (sales volume).

## Publication and Abstracts:

Grazier, C., and Torres, J.A. 1990. The impact of varied cooling rates on Cheddar cheese quality. Presented at the 79th Annual Oregon Dairy Industries Conference, Feb. 13-14, Eugene OR.

Grazier, C., Simpson, R., McDaniel, M.R., Bodyfelt, F.W., Torres, J.A. 1990. Temperature effects on the microbial and sensory properties of Cheddar cheese. To be presented at the 51st Annual Meeting of the Institute of Food Technologists, June 17-20, Anaheim, CA.

Grazier, C., McDaniel, M.R., Bodyfelt, F.W., and Torres, J.A. Temperature of maturation effects on the sensory properties of Cheddar cheese. *J. Food Sci.* (In review).

Grazier, C., Simpson, R., Bodyfelt, F.W., and Torres, J.A. Temperature effects on the microbial activities occurring during cooling and ageing of Cheddar cheese. *J. Food Sci.* (In preparation).

**Project Title:** Cooling rate of Cheddar cheese: Comparison between 40 and 640 lb blocks and uniform cooling of 640 lb blocks

**Personnel:** J.A. Torres, Assistant Professor, Food Science and Technology, Oregon State University

F.W. Bodyfelt, Professor, Food Science and Technology, Oregon State University

C. Grazier, Graduate Research Assistant, Food Science and Technology, Oregon State University

R. Simpson, Graduate Research Assistant, Food Science and Technology, Oregon State University

J. Bouzas, Graduate Research Assistant, Food Science and Technology, Oregon State University

Conly L. Hansen, Associate Professor, Dept. of Nutrition and Food Sciences, Utah State University

**Funding Sources:** Western Dairy Foods Research Center  
Tillamook County Creamery Assoc. (Tillamook, OR)  
Oregon State University Agricultural Experiment Station  
Utah Agricultural Experiment Station

### Objectives:

The goal of this research project is to suggest cooling process changes to achieve a better temperature control and temperature uniformity during the cooling of 640 lb Cheddar cheese blocks.

The specific objectives of this research project are:

1. To quantify the effect of heterogeneous temperature distributions on the residual starter culture activity and the growth of adventitious microorganisms during aging of 640 lb cheese blocks.
2. To quantify the effect of heterogeneous temperature distributions and microbial activity on the chemical and sensory characteristics of 640 lb cheese blocks.
3. To implement a heat transfer model for the cooling of 640 lb cheese blocks.
4. To implement a combined model for heat transfer and microbial activity of residual starter and typical adventitious microorganisms.
5. To use a mathematical optimization method to determine cooling conditions that reduce sensory differences between the various locations in a 640 lb cheese blocks.

## Results:

The experimental procedures used in this research were developed primarily to accommodate the large experimental error associated with sensory analysis. The design includes four batches with two replications at each of four temperature treatments sampled throughout the ripening process. Samples were drawn from commercial production after the pressing step in the process used at Tillamook County Creamery Assoc. (Tillamook, OR). Blocks were cut and vacuum packaged under sanitary conditions. Batch collection was timed for early and late lactation to take advantage of maximum variability among batches. Samples were stored under constant temperature conditions (5°C, 15°C, 25°C and 35°C). Sample size varied according to analysis need (microbiology, chemistry and sensory). In all cases, samples were small enough to reach storage temperature within 1-2 hours.

### Heat Transfer Modelling

A computer program for the heat transfer model described in the proposal has been implemented for the cooling of single blocks (any size and shape). The model takes into account the effect of the thermal properties of the cheese and the packing materials (one or more materials: wood, metal or plastic) and has been validated using data obtained at Tillamook County Creamery Assoc. and that published by Reinbold and Ernstrom (1). Further tests will be conducted at OSU and USU to make it available to dairy processors.

### Microbiological Analysis

Several elements of the manufacturing process affect the types and quantities of bacteria present in the cheese as it goes into cooling and ripening: (a) pH of the curd; (b) the amount and timing of the salting; (c) the composition and inoculum level of the starter culture; (d) sanitary conditions of the equipment and the raw materials as well as the extent of the heat treatment of the milk before use. At Tillamook County Creamery Assoc., milk used for Cheddar cheese is flash heated and not pasteurized.

Initially, the starter culture can be found in the aging cheese microflora in the greatest numbers (Fig.1). In Cheddar, this is likely to be *Lactococcus cremoris*. Within the first few days, a slight amount of growth will be observed. This is followed by a rapid die-off of cells primarily due to a continued drop in the pH of the cheese. Enzymes of both viable and dead starter cells are involved in flavor development. Starter bacteria which reach too high a level or survive too long have been associated with production of bitter flavor defects.

In cheese produced under good manufacturing conditions, the initial counts of non-starter organisms are low (Fig.1). Of the adventitious organisms, the lactobacilli are well represented. Characteristic Cheddar flavor can be attained without the presence of the adventitious organisms, but is achieved more rapidly when they are present.

Enumeration of the non-starter fractions of bacteria present in Cheddar cheese is straightforward using selective or indicative media. The starter bacteria is a different problem because they require the use of non-selective medium and isolation of individual colonies for further characterization (morphological and biochemical). This procedure is time-consuming, expensive and most of the times inconclusive. Development of the API system has facilitated this identification but remains an expensive procedure.

Samples collected at Tillamook were analyzed for total plate counts, coliforms, lactobacilli and non-starter lactobacilli. Our analysis of microbiological data has concentrated on the non-starter lactobacilli counts as a function of time and storage temperature. At 5°C, there is essentially no change in microbial counts for up to 80 days (Fig. 2). It is interesting to note that this sample could be used as a reference sample where the lack of microbial growth should be reflected in specific chemical and sensory properties. At 15°C there is significant growth up to day 40 (Fig. 3)

At 25°C there is significant growth up to day 16 (Fig.4), and at 35°C the growth phase seem to end at day 6, after which significant cell count reduction occur (Fig. 5) . A statistical analysis of the growth data suggested the need to modify our sampling plan to include 6-10 cheese samples during the exponential growth phase.

Two theoretical models can be used to analyze the exponential growth phase: the first order and the Verhulst equation (eqs . 1 and 2, respectively) .

$$dN = \mu N dt \quad (1)$$

$$dN = \mu [(b - N)/b] N dt \quad (2)$$

where  $N$  = number of microbial counts,  $\mu$  = specific growth rate,  $t$  = time, and  $b$  = maximum microbial counts. The effect of temperature on the specific growth rate,  $\mu$ , can be analyzed with either one of the following three models:

$$\mu = \mu_0 \exp [ - E_a/RT ] \quad (3)$$

$$\mu = \mu_0 \exp (1 + cT) \quad (4)$$

$$\mu^{1/2} = b (T - T_0) \quad (5)$$

where  $\mu_0$ ,  $c$ ,  $b$  are constants,  $E_a$  = energy of activation for growth,  $R$  = universal gas constant,  $T$  = absolute temperature and  $T_0$  = theoretical minimum growth temperature. A preliminary analysis of the effect of temperature on the specific growth rate gave an estimate for the energy of activation of about 16 Kcal/g-mole. Additional experiments at other storage temperature conditions (12°C and 20°C) are needed to confirm this activation energy value and the suitability of the Arrhenius model as compared to the other available models (eqs. 4 and 5). However, this low activation energy value would suggest that microbial growth of non-starter lactobacilli could be controlled by the diffusion of nutrients in Cheddar cheese (see Table 1).

Enumeration of the starter culture has presented a few experimental problems. New batches will be enumerated using a three step approach: (1) enumeration on a semi-selective medium (betaglycerophosphate); (2) pure culture isolation and API characterization at 4 representative time points for each temperature treatment; and (3) extrapolation of the semi-selective medium API test results to the results obtained at other times.

### Chemical Analysis

Chemical analysis requirements for the completion of this project include: carbohydrate and organic acid analysis by HPLC, D-L lactic acid determination by an enzymatic method (5,6), degree of proteolysis by a trinitrobenzenesulphonic acid spectrophotometric method (7), pH, and titratable acidity. Salt concentration, fat, moisture and total protein content were thought to be needed only at time 0. However, preliminary experiments show significant syneresis during storage, suggesting the need for more close monitoring of these values.

We chose the following methodology for the carbohydrate and organic acid analysis of Cheddar cheese (2,3,4). Samples are extracted using an acetonitrile:water (ratio needs to be optimized), the mixture is centrifuged at 1600 x g and the supernatant filtered through a regenerated cellulose filter. Ten  $\mu$ l of the filtered solution will be injected into a Bio-Rad Aminex HPX-87H column with a Cation-H<sup>+</sup> Microguard column. The mobile phase to be used is 0.0090 N H<sub>2</sub>SO<sub>4</sub> (flow rate of 0.8 ml/min). Variable wavelength UV and refractive index detectors, installed in tandem, will be used for the quantification of organic acids and carbohydrates, respectively. Preliminary runs using pure carbohydrate and organic acid solutions gave good separations of major components (Fig.6).

### Sensory Analysis

A descriptive panel has been trained and an appropriate set of aroma and flavor-by-mouth descriptors have been identified. Sensory testing has been conducted on samples from two batches pulled from manufacturing dates in January. Sensory testing was done at 90 and 120 days into the ripening process for both batches. At 90 days, the cheese ripened at 35°C was unpalatable and was not presented to the panel. The 5°C, 15°C, and 25°C cheeses were scored using 20 different aroma and flavor descriptors (Fig.7) and a 15 point intensity scale (Fig.8). No significant differences were observed between batches. At 90 days, significant differences between temperature treatments were as follows (Tables 2 and 3):

- 1) Overall intensity of both flavor and aroma, pungent acidic and pungent sulfur aroma, and goaty flavor were greater for cheeses ripened at higher ripening temperatures.
- 2) Buttery aroma and flavor were significantly lower in the 25°C cheese compared to the 5°C and 15°C cheeses.
- 3) Fruity, goaty, dirty, and yeasty aroma and sour, salty, bitter, fruity, dirty, and sulfur flavors were significantly greater in cheese ripened at 25°C

At 120 days similar trends were observed with the following exceptions:

- 1) Goaty and dirty aroma, and dirty and sulfur flavor were greater at higher ripening temperatures. 2) Sour and salty flavors were significantly lower in the 5°C cheese compared to the 15°C and 25°C. 3) The 25°C cheese was significantly sweeter than the 5°C cheeses. In comparing 90 and 120 day cheese within each temperature treatment, differences were found only in cheese ripened at 25°C.
- 2) Overall intensity of flavor and aroma increased significantly.
- 3) Nutty aroma decreased.
- 4) Dirty aroma, and dirty and sulfur flavors increased.

In general, panelist by treatment interaction was minimal where significant differences in descriptor scores were detected. This allowed us to be confident in the ability of the panelists to describe the cheese characteristics consistently. The only exception to this was in the scoring of sour flavor where one panelist scored markedly different from the others. Review sessions emphasizing the scoring of the sour flavor will be conducted before further testing.

Considering these findings, we have determined that more and earlier analysis is indicated for future batches. Later batches will be tested at 7, 30, and 60 days in addition to 90 and 120 days. Consent of the Human Subjects Board has been obtained to test before the legal minimum of 60 days for cheese made with unpasteurized milk.

### **Impact of Research:**

American is the major type of cheese produced in the United States (2.8 billion pounds in 1986). The vast majority of this production is Cheddar cheese. Unfortunately, there are wide fluctuations in the sensory quality of individual lots of Cheddar cheese. Conservatively, experienced cheese graders frequently categorize 30-40% of all American Cheddar cheese as being "high acid (sour)" or "bitter" in off-flavor.

One of the processing steps believed to be responsible for the lack of uniformity in Cheddar cheese quality is cooling of the cheese block before aging. This research project examines the microbiological, chemical and sensory consequences of lack of temperature control during this processing step and will apply engineering principles to suggest cooling conditions leading to more uniform and consistent quality.

We believe that Cheddar cheese sales are not increasing in great part because of the high variability in product quality. The economical impact is enormous because: (a) much Cheddar cheese has to be marketed at a younger age than is perhaps optimal for best price; (b) the frequency of the sourness/bitterness defect reduces its quality image and hence reduced its market value (price/unit) and also limits its overall consumer acceptance (sales volume).

## References:

1. Reinbold, R.S. and Ernstrom, C.A. 1988. Effect of non-uniform cooling on moisture, salt, and pH distribution in 290-kg blocks of stirred-curd Cheddar cheese. *J. Dairy Sci.* 71:1499.
2. Marsili, R.T., Ostapenko, H., Simmons, R.E. and Green, D.E. 1981. High performance liquid chromatography determination of organic acid in dairy products. *J. Food Sci.* 46:52.
3. Pirisino, J.F. 1983. High performance liquid chromatographic determination of lactose, glucose, galactose in lactose-reduced milk. *J. Food Sci.* 48:742.
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6. Gawehn, K. and Bergmeyer, H.U. 1974. D ~ Lactate determination with lactate dehydrogenase and NAD. In *Methods in Enzymatic Analysis*, Bergmeyer, H.U. (ed.), 2nd edition, Vol.3, pp.1492. Academic Press, NY.
7. Polychroniadou, A. 1988. A simple procedure using trinitrobenzenesulphonic acid for monitoring proteolysis in cheese. *J. Dairy Research* 55:585.

TABLE 1  
Typical  $E_a$  values, Kcal/mol

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Diffusion	0 - 15
Microbial growth	20 - 60
Enzyme reactions	10 - 15
Hydrolysis	15
Lipid oxidation	10 - 25
Nutrient losses	20 - 30
Non-enzymatic browning	25 - 50
Spore destruction	60 - 80
Vegetative cell destruction	50 - 150
Protein denaturation	80 - 120





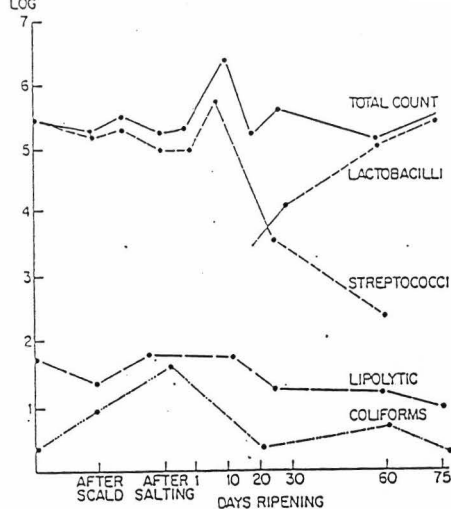


FIGURE 1. Typical Microbial Activity During Processing and Aging of Cheddar Cheese

CFU

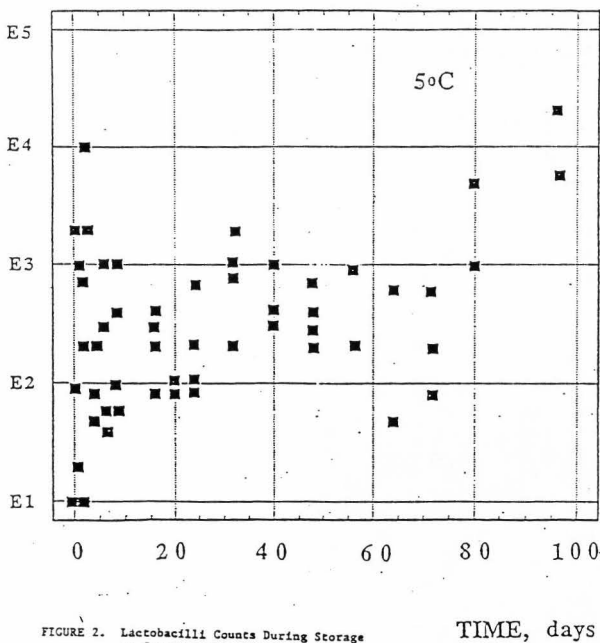


FIGURE 2. Lactobacilli Counts During Storage at Constant Temperature

TIME, days

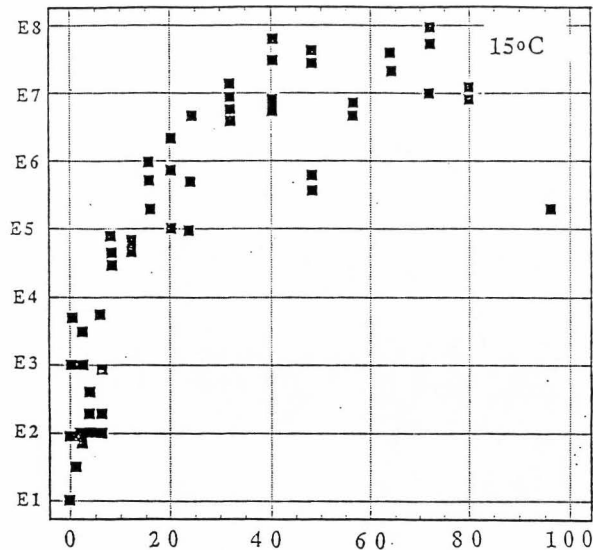


FIGURE 3. Lactobacilli counts during storage at constant temperature

TIME, days

CFU

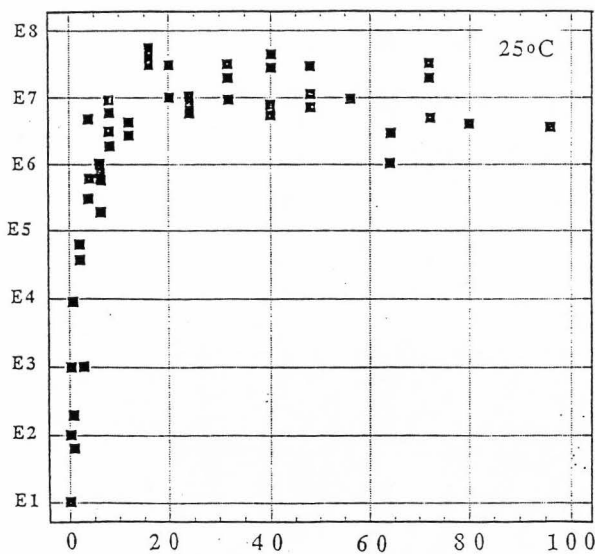


FIGURE 4. Lactobacilli Counts During Storage

TIME, days

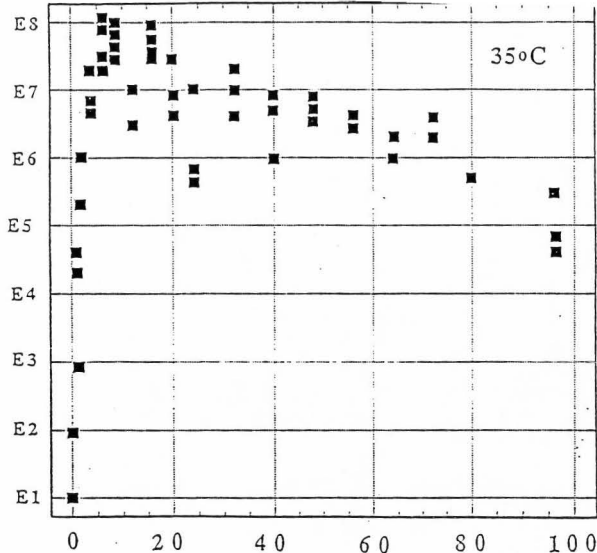


FIGURE 5. Lactobacilli Counts During Storage at Constant Temperature

TIME, days

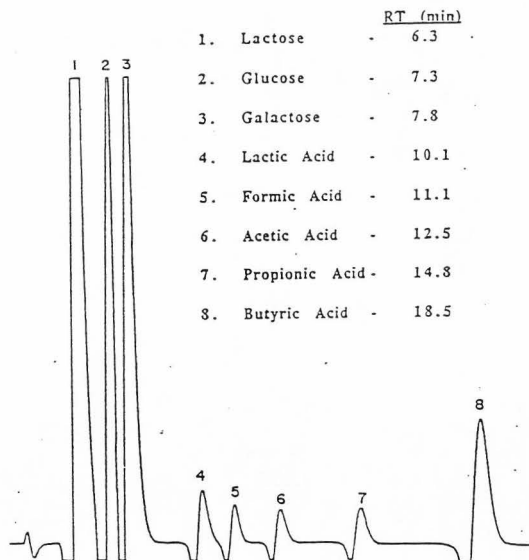


FIGURE 6. HPLC run of an aqueous solution of organic acids and carbohydrates

Assay conditions: Bio-Rad Aminex HPX-87H column w/ Cation-H<sup>+</sup> Microguard.

	<u>Sample #</u>	<u>Sample#</u>
AROMA		
Overall Intensity	_____	_____
Nutty	_____	_____
Fruity	_____	_____
Pungent (acidic-formic)	_____	_____
Pungent (sulfur)	_____	_____
Goaty (butyric)	_____	_____
Dirty (isovaleric)	_____	_____
Yeasty	_____	_____
Other (please list)	_____	_____
FLAVOR		
Overall Intensity	_____	_____
Sour	_____	_____
Salty	_____	_____
Bitter	_____	_____
Sweet	_____	_____
Buttery	_____	_____
Nutty	_____	_____
Fruity	_____	_____
Goaty (butyric)	_____	_____
Dirty (isovaleric)	_____	_____
Sulfur	_____	_____
Other (please list)	_____	_____

FIGURE 7. Aroma and Flavor-by-mouth Descriptors used by the Trained Panel

## DESCRIPTIVE CHEDDAR CHEESE PANEL

### Scoring Procedure

0 = None	8
1 = Just detectable	9 = Moderate to large
2	10
3 = Slight (oil)	11 = Large ("grape" drink)
4	12
5 = Slight to moderate	13 = Large to extreme
6 = Sour, bitter, salt solutions	14
7 = Moderate ("orange" drink)	15 = Extreme ("Cinnamon" Gum)

FIGURE 8. Scoring Performance Used by a Trained Panel

**Project Title:** Cooling rate of Cheddar cheese: comparison between 40 and 640 lb blocks of uniform cooling of 640 lb blocks

**Personnel:** J. Antonio Torres, Principal Investigator, Dept. Food Sci. & Technology, Oregon State University

Floyd W. Bodyfelt, Principal Investigator, Dept. Food Sci. & Technology, Oregon State University

Conly L. Hansen, Principal Investigator, Dept. Nutrition & Food Sciences, Utah State University

Jorge Bouzas, Ph.D. Graduate Student, Dept. Food Sci. & Technol., OSU

Connie Grazier, M.S. Graduate Student, Dept. Food Sci. & Technol., OSU

**Funding:** Western Dairy Foods Research Center  
Tillamook County Creamery Assn. (Tillamook, OR)

### **Objectives:**

In spite of major research and process improvement efforts, there is still a wide variation in the sensory properties of the most popular cheese variety consumed, Cheddar cheese. Cooling of the freshly formed cheese is believed to be a processing step requiring closer control to achieve uniform and consistent flavor quality. The effect of time and temperature on Cheddar flavor quality was investigated on the basis of the following objectives:

1. To develop a computer program that calculates cooling rate of 40 lb and 640 lb blocks as a function of cooling conditions and Cheddar cheese composition.
2. To demonstrate that a more consistent quality can be achieved by controlling the cooling rate of 40 and 640 lb Cheddar cheese blocks.
3. To evaluate sensory properties and chemical composition of a commercial mild Cheddar cheese.
4. To identify temperature conditions leading to sensory characteristics similar to commercial Cheddar cheese and confirmed by chemical and microbial analysis.
5. To develop a mathematical model combining heat transfer calculations and kinetic expressions for the effect of time and temperature on selected sensory and chemical indexes.

6. To use the model developed in (5) to determine the cooling rate and aging room temperature conditions that result in the quality identified in (4) in a uniform manner for the entire cheese block.
7. To quantify the effect of heterogenous temperature distributions and microbial activity on the chemical and sensory characteristics of 640 lb cheese blocks.

## Results:

The experimental procedures used in this research were developed primarily to accommodate the wide range of temperature effects to be covered (5-35°C) and the large experimental error associated with sensory analysis. Samples were obtained from the production of a commercial cheese manufacturer (Tillamook County Creamery Assn., Tillamook, OR) directly after the pressing operation. Forty pound blocks from the same vat lot were cut into 2.5 cm x 5 cm x 5 cm pieces. Each piece was vacuum shrink-wrapped in commercial O<sub>2</sub>-barrier cheese film. Samples were small enough to reach storage temperature within 1-2 hours. Samples were randomly assigned to the five storage temperatures: 12, 15, 20, 25, and 35°C and tested at different stages during a three month period. Four batches with one replicate were used for sensory, microbial and chemical analysis tests. The following were the most significant findings:

1. Microbial, chemical and sensory analysis of samples stored at constant temperature for a three month period confirmed the importance of early temperature control to avoid flavor quality problems.
2. A kinetic analysis of microbial growth data suggests that microbial activities are controlled by diffusion phenomena involving the movement of nutrients and/or the diffusion of metabolic waste from the bacterial cell. This finding needs to be analyzed within the context of the temperature values observed during cooling and aging. Cheese blocks cooled at a slow rate have a high temperature during the first days of the ripening process and cause a rapid die-off of the starter culture. Faster cooling slows down the activity of the starter culture and this allows the starter culture to remain active for a longer period of time without reaching inhibitory conditions in the microenvironment surrounding the cell. A more extensive lactose utilization by the starter culture would lead to a reduction of the flavor quality problems associated with the growth of adventitious microorganisms.
3. Simulations using a computer model combining heat transfer calculations with kinetic equations for sensory and chemical changes during early ripening identified a large number of cooling (time and air temperature) and aging (air temperature) conditions leading to similar values for the chemical and sensory indexes used as flavor quality indicators. This observation implies that the optimum operating conditions for the cooling and aging process covers a range of temperatures and is not constrained to a single operating point.

## **Impact of research:**

This project examined the sensory, chemical and microbial changes involved in the process to convert the rubbery, relatively flavorless matrix of fresh pressed curd into Cheddar cheese with distinct aroma, taste, body, and texture. For Cheddar cheese a period of at least 5 to 10 months refrigerated storage is required, during which operating costs and interests on capital involved in cheese aging significantly add to the cost of production. Our results have been used to develop models of the aging process to select cooling and aging conditions leading to improved and consistent flavor quality. Conversations with personnel at Tillamook County Creamery Assn. who have installed new equipment for the rapid cooling of cheese have confirmed predictions made on the basis of our experimental results.

## **Publications:**

Bouzas, J., Bodyfelt, F.W., and Torres, J.A. 1991. A potential analytical assessment of Cheddar cheese flavor defects. *Int. Dairy J.*:IN PRESS.

Bouzas, J., Kant, C., Bodyfelt, F.W., and Torres, J.A. 1991. Simultaneous determination of sugars and organic acids in Cheddar cheese by HPLC. *J. Food Sci.* **56**(1):276-278.

Grazier, C., McDaniel, M.R., Bodyfelt, F.W., and Torres, J.A. 1991. Temperature effects on the sensory properties of Cheddar cheese. *J. Dairy Sci.*:IN PRESS.

Bouzas, J., Kant, C.A., Bodyfelt, F.W., and Torres, J.A. Characterization and interpretation of time-temperature effects on chemical changes occurring during Cheddar cheese aging. *Int. Dairy J.* (IN REVIEW)

Bouzas, J., Roncagliolo S., Almonacid-Merino, S.F., Banga, J.R., Simpson, R., and Torres, J.A. 1991. Simulation and selection of operating conditions for the cooling and aging of 40 lb Cheddar cheese blocks. *Food and Bioproducts Processing.* (IN REVIEW)

Grazier, C.L., Simpson, R., Roncagliolo, S., Bodyfelt, F.W., and Torres, J.A. Temperature effects on non-starter bacteria populations during cooling and aging of Cheddar cheese blocks. *Int. Dairy J.*



**Project Title:** Rapid and Uniform Cooling of Cheddar Cheese Blocks

**Personnel:** Conly L. Hansen, Dept. of Nutrition and Food Sciences, Utah State University.

Sterling J. Larsen, Dept. of NFS, USU

Antonio Torres, Oregon State University

**Funding:** Western Dairy Foods Research Center

### **Objectives:**

In researching the cause of high acid quality defects in American style Cheddar cheese, Reinbold and Ernstrom (1) monitored moisture, salt, and acidity levels at numerous locations within large (640 lb.) Cheddar cheese blocks. Their results show significant variation of moisture, salt, and acidity levels according to position in the block. Moisture level differences were most significant, averaging 5.73% less in the center than at the side when the cheese block was cooled at 5°C. When the block was cooled at 22°C, moisture level differences averaged 0.96%. Salt and acidity levels followed a similar trend with the center averaging lower levels than side locations.

Temperature profiles from cheese blocks cooled at 5°C and 22°C suggest that thermal gradients within the cheese block could be the driving force for moisture transfer from the center of the block to the sides. Mass transfer by means of a temperature gradient is referred to as thermo-osmosis. Collins (2) described thermo-osmosis as moisture movement through porous solids by three possible mechanisms. First, vaporization in the hotter central region occurs, vapor transfer through the medium follows, and the condensation in the colder exterior regions. Second, liquid transfer by vapor pressure differences occurs, vapor pressure being directly related to temperature. Finally, liquid transfer by capillary pressure gradients occurs due to surface tension temperature dependence. Surface tension varies inversely with temperature. It was also shown that moisture levels stabilize within 48 hours of cooling initiation.

1. Characterize the link between moisture movement within large Cheddar cheese blocks to the cooling rate and temperature uniformity of the block.
2. Determine the thermal conductivity of fresh Cheddar cheese curd and compare the value with the thermal conductivity of aged Cheddar cheese.

3. Develop a computer model to predict internal cheese block temperature as a function of position and time ( $T(r,z,t)$ ,  $T(x,y,z,t)$ ) when exposed to various cooling situations.

### Results:

By reversing the cooling scenario and monitoring the moisture level response, it was observed that moisture levels followed the reversed temperature profiles. Moisture was significantly greater at the colder central region and less at the warmer exterior locations. When temperature gradients were less than  $7-8^{\circ}\text{C}$  the moisture gradients were less than 1.5%.

A computer model for a cylindrical cheese block has been developed using an explicit finite difference method (backward difference). Modifications are underway to improve the accuracy of the program by using an implicit finite difference method (forward difference) and removing uncertain convection terms.

Values for thermal conductivity of Cheddar cheese are tabulated for cured cheese but no values are given for fresh curd. The thermal conductivity probe to be used for these measurements has not yet been received. Completion of these tests is expected within the next month. Determination of possible thermal conductivity variation during initial cooling will also remove variation from the computer model.

### Impact of Research:

Knowledge that large temperature gradients cause moisture transfer in large cheese blocks will allow possible modifications to the present cooling techniques to diminish moisture movement. Application could also be made to other porous products that undergo large thermal variation for extended lengths of time.

### Publications:

- Reinhold, R. S., and C. A. Ernstrom. 1988. Effect of nonuniform cooling on moisture, salt, and pH distribution in 290-kilogram blocks of stirred-curd Cheddar cheese. *J. Dairy Sci.* 71:1499.
- Collins, R. E. 1961. Flow of fluids through porous materials. Reinhold Publ. Corp., New York, NY.

**Project Title:** A new method for measuring syneresis of renneted gels applied to development of cheese.

**Personnel:** Conly L. Hansen, Professor, Nutrition and Food Sciences, Utah State University.

**Funding Sources:** Western Dairy Foods Research Center  
National Dairy Promotion and Research Board

### **Objectives:**

The primary objective is to develop a rapid, inexpensive, accurate, real time method of measuring syneresis.

#### Specific Objectives

- 1) Determine the necessary optical components and the relative optimal spacing.
- 2) Design and calibrate the sampling vial for the desired optical characteristics.
- 3) Create a program that will use the measured amount of light scattered to compute the particle size.
- 4) Determine the optimal angle for collection of scattering data.
- 5) Make some measurements of syneresis with this method.

### **Results:**

New Project. We have bought the laser, lens and assorted hardware and are now building the device. At the same time the graduate student on the project has started writing the code to convert light readings into curd size.

### **Impact of Research:**

Syneresis is important in many dairy products and especially in the manufacture of cheese. It is the underlying physical mechanism that produces cheese from milk. Over the years there have been many projects devoted to understanding syneresis. The secrets of syneresis still elude researchers (Walstra et al., 1985). Today the factors that affect syneresis are understood better than ever before but syneresis itself is still some what of a mystery. The major reason for this is the inability to make accurate measurements (Fox, P. F., 1987). This factor permeates all research on syneresis and the factors that affect it. If the syneresis of curd particles were measured accurately we would be able to understand syneresis better and make inroads into controlling it. By controlling it we could better control the quality and optimize the quantity of cheese produced, we could improve shelf life of dairy products and insure the product was more appealing at the time of sale, and we could create new products by altering the process during manufacture.

To control syneresis you must understand it first. Knowing the rate of syneresis is inherent to understanding it. Over 400 methods have been tried to accurately measure the rate of syneresis and there are inaccuracies inherent in each (Stoll, W.F., 1966). The properties of the surrounding media can affect the rate of syneresis (Walstra et al., 1985).

Still another family of methods depends upon a dilution of a substance in the whey. After the curd is formed this tracer is added to the whey. At time intervals samples are drawn and the concentration of the tracer is measured. But this too has inherent inaccuracies as invariably some of the tracer is absorbed into or bound onto the curd itself (Zviedraus, P. and Graham, E.R.B., 1981), e.g. cheese made from goats milk (Nilsen, K.O. and Abrahamson, R.K., 1985).

A recent approach proposed by Pearse et al. (1985) utilizes the optical properties of the whey as a measure of syneresis. Samples of the whey drawn at intervals shows that the optical characteristics change with time. This is what would be expected as the concentration of whey solids is diluted. But their method is not free of errors (Pearse, et al., 1984).

And with all of the above methods time, is a major factor. A lot of time is required to run an analysis on a batch and hence makes an exhaustive analysis of even one factor difficult (Ernstrom, C.A., 1988). But the method we propose will be much faster and more accurate.

This method will use the light scattering characteristics of the curd itself to analyze the curd size. This method will not have the drawback of being absorbed into the curd as in the tracer method, or the inconvenience of having to separate the curd from the whey for measuring. By making more measurements in a run, the size of the curd vs. time may be plotted and a rate of shrinkage can be calculated. Since the curd is not removed from the whey, and since the environment can remain intact the effects of the measurement on the curd will be minimal. This method will give us a much more accurate picture of the rate of syneresis.

This technology is new to the dairy industry and may prove valuable in many areas. It may eventually provide a method for measuring the size of proteins and fat structures in milk. And related technologies may help in analysis of moisture content, fat and solids not fat in a quicker fashion.

Yields, again, were not significantly different (see charts 1 and 2). The ropy vat did show improved protein recovery, but not enough to cause a significant increase in yield.

#### **Impact of Research:**

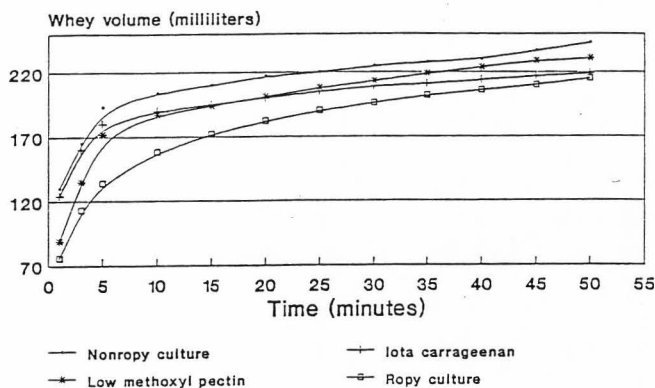
1. Potential method for moisture control in milk gels which have experienced various treatments affecting water holding capacity.
2. Incorporation of polysaccharide material into the cheese curd may improve body and texture. At this point, it is not apparent that polysaccharide incorporation has been accomplished. Body and texture effects are being evaluated.

#### **Publications:**

Olsen, R. L. 1989. Effect of exopolysaccharides from *Streptococcus thermophilus* on mozzarella cheese yield. J. Dairy Sci. 71(Suppl. 1). Manuscript in preparation.

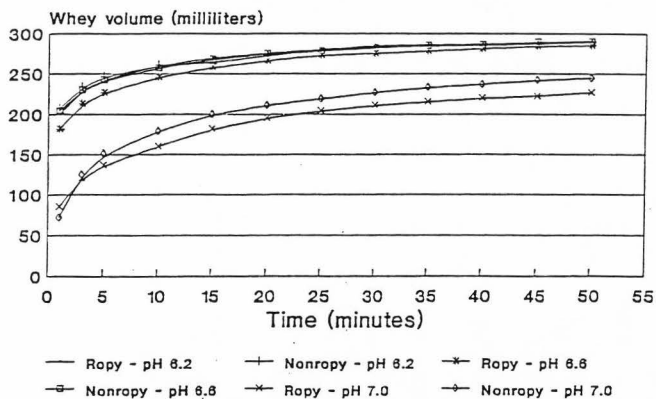
Figure 1. Effects of polysaccharide materials on syneresis with ropy and nonropy cultures

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Graph 1

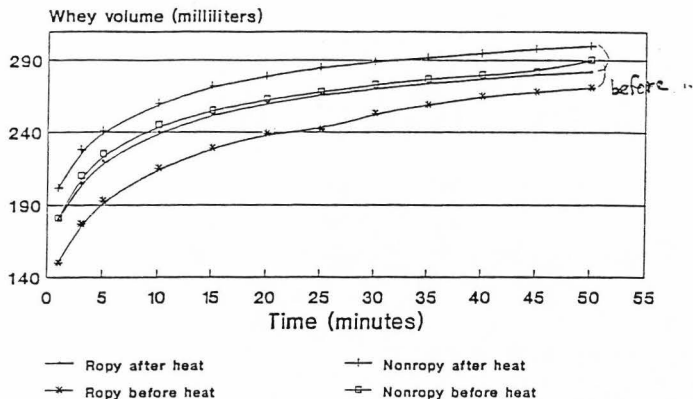
Effects of pH on syneresis using ropy and nonropy cultures



Graph 2

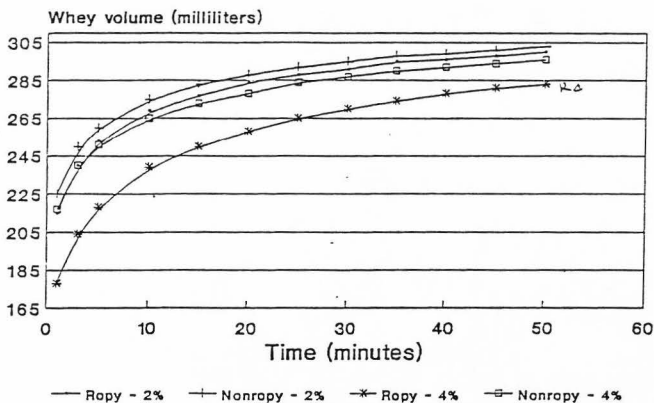
## Effects of heat on syneresis using ropy and nonropy cultures

92



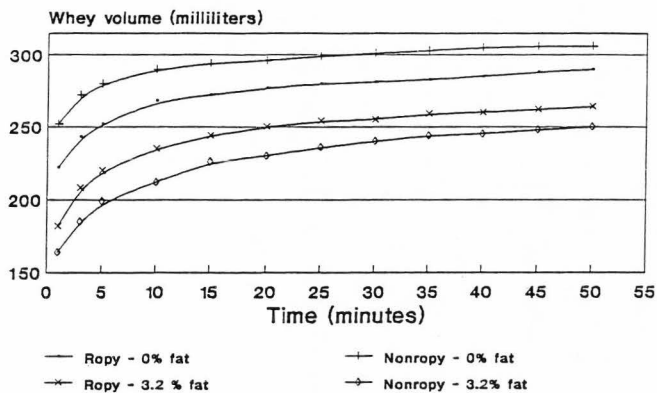
Graph 4

## Effects of culture level on syneresis using ropy and nonropy cultures



Graph 3

# Effects of fat on syneresis using ropy and nonropy cultures



Graph 5



CHEESE YIELD DATA (LARGE TRIAL)

	<u>ROPY</u>	<u>NON-ROPY</u>
Unadjusted Yield	7.7	7.7
Adjusted Yield (45% H <sub>2</sub> O)	8.3	8.3

Chart 1

NUTRIENT RECOVERY (LARGE TRIAL)

	<u>ROPY</u>	<u>NON-ROPY</u>
Protein	71.3	70.5
Fat	88.1	88.3

Chart 2

**Project Title:** Improvement of Mozzarella cheese yield and physical properties through proteinase modification of starter cultures

**Personnel:** Gary H. Richardson, Professor, Nutrition and Food Sciences, Utah State University

Craig J. Oberg, Research Associate, Nutrition and Food Sciences, Utah State University

Lynn V. Moves, Research Technician, Nutrition and Food Science, Utah State University

Amos Wang, Graduate Assistant, Nutrition and Food Science, Utah State University

**Funding Sources:** Western Dairy Foods Research Center  
Utah Agricultural Experiment Station.

#### Objectives:

1. Find proteinase negative (Prt<sup>-</sup>) thermolactic cultures that can be used to manufacture Mozzarella cheese and improve the physical properties of curd without causing adverse flavor reactions.
2. Screen the available proteinase negative strains of *S. cremoris* for acid production and survival at the Mozzarella cooking temperatures.
3. Isolate and test proteinase negative strains of *S. thermophilus* and *L. bulgaricus* for casein proteolysis and acid production. Trial production studies will follow using direct acid Mozzarella curd as the control.
4. Obtain current physical testing procedures from Pizza Hut and other Mozzarella cheese buyers, select representative methods, and quantitate the changes caused by the proteinase systems involved. The physical properties of the cheese to be measured will include stretchability, cook color, and meltability.

#### Results:

1. A number of Prt<sup>-</sup> strains of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* were found and evaluated in Mozzarella cheese production.
2. Activity tests were run at 37, 42, 44, and 46°C on the following proteinase negative strains of *Streptococcus cremoris*: UC 320 Prt<sup>+</sup>, UC 320 Prt<sup>-</sup>, UC 85 Prt<sup>+</sup>, UC 45 Prt<sup>-</sup>, and UC 310 Prt<sup>-</sup>. Results showed noticeable activity up to 42°C for several strains. High temperature isolation of *S. cremoris* UC Prt<sup>-</sup> strain was also done. Results showed only UC 85 Prt<sup>-</sup> grew at all the temperatures tested, and that UC 310 Prt<sup>-</sup> and UC 161 Prt<sup>-</sup> grew well at 39°C but not at higher temperatures. Mozzarella cheese could be manufactured at a lower temperature (39°C) using these organisms. High temperature incubation of *S. cremoris* Prt<sup>-</sup> cultures at 42°C was done to

select for cultures that could adapt to elevated temperatures. Only UC 85 Prr<sup>-</sup> exhibited growth at this temperature and no new isolates were found.

3. A large number of strains of both thermolactic organisms were screened for proteolytic ability with the OPA test. These cultures were also analyzed by amino acid analysis which was found to provide a much more detailed profile of their proteolytic characteristics. A number of Prr<sup>-</sup> strains of both *S. thermophilus* and *L. bulgaricus* were characterized for both proteolysis and acid production. Mozzarella cheese was manufactured using these cultures, their Prr<sup>+</sup> parents, and the direct acid method. The physical properties of these cheeses were then compared over a 28 d period.
4. The curd was evaluated using methods to measure color changes and meltability during heat treatment. A Minolta reflectance color meter was used and the b values were used to measure the darkening that occurred with heat treatment. A tube method was used to evaluate the meltability of the curd upon heating. Differences were found that proved to be strain dependent. (Figure 1 and Figure 2) A low viscosity Brookfield viscometer was combined with a Helipath stand to allow objective measurements of the rheology, specifically stretchability, of the curd at 60° (Figure 1 and Figure 2).

#### Impact of Research:

The second leading cheese produced in the U. S. is Mozzarella cheese. Proteinase negative starter cultures used in the production of Cheddar cheese, cottage cheese, and acid casein have been shown to increase yield, along with providing a number of other advantages. If proteinase negative cultures could be used in the production of Mozzarella and other high temperature Italian cheeses, an increase in yield may be possible. Additionally, if the effects of proteinase activity can be measured, there may be a possibility of improvement in the stretching, along with other physical properties. We need to learn more about the effect proteinase activity of the thermolactic cultures have on the physical qualities of these cheese types. The major purchasers of pizza cheese are concerned that the physical qualities of Mozzarella curd begin to deteriorate at about ten days of age. The proteolytic activity of the starter culture is thought to be the cause of reduction of stretch. By incorporation of less proteolytic thermolactic starter cultures, we desire to extend these superior qualities. The better and more consistent the physical properties, the more sales for the product.

#### Publications:

1. Oberg, C.J., A. Wang, L.V. Moyes, and G.H. Richardson. 1989. Effect of proteolytic activity of thermolactic cultures on physical properties of Mozzarella cheese. *J. Dairy Sci.* (Abstract accepted for Annual meeting) (Manuscript in preparation)
2. Weimer, B.C., C.J. Oberg, L.V. Moyes, R.J. Brown, and G.H. Richardson. 1989. Comparison amino acid analysis and o-phthalaldehyde methods to characterize proteolysis by *Lactobacillus bulgaricus*. *J. Dairy Sci.* (submitted).
3. Oberg, C.J., B.C. Weimer, L.V. Moyes, R.J. Brown, and G.H. Richardson. 1989. Proteolytic characterization of *Lactobacillus bulgaricus* strains by amino acid analysis. *J. Dairy Sci.* (In preparation).
4. Oberg, C.J., L.V. Moyes, and G.H. Richardson. 1989. Acid production of *Streptococcus cremoris* in curd made at elevated temperatures. *J. Dairy Sci.* (In preparation)

FIGURE 1

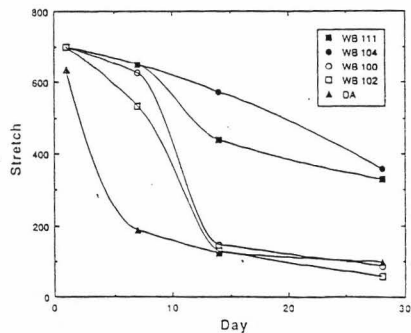
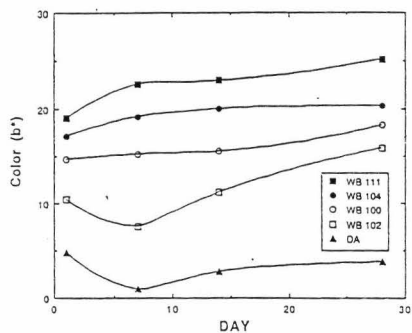
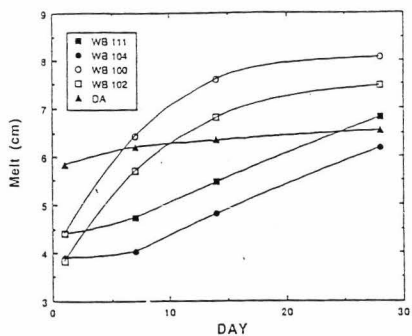
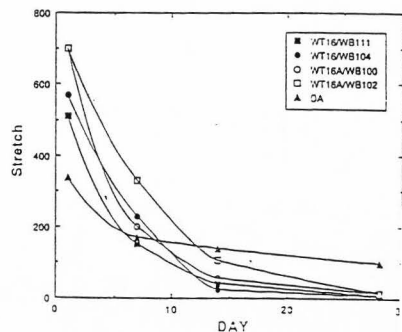
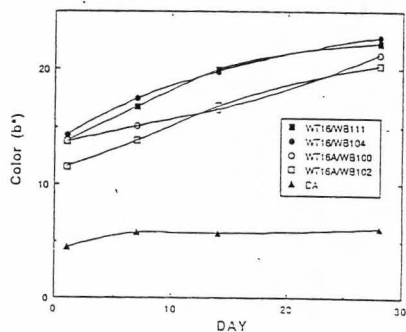
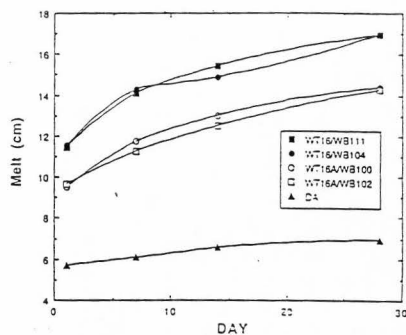


FIGURE 2



**Project Title:** Improvement of Mozzarella cheese yield and physical properties through proteinase modification of starter cultures

**Personnel:** Gary H. Richardson, Professor, Dept. of Nutrition and Food Sciences, Utah State University.

Craig J. Oberg, Research Associate, Dept. of NFS, Utah State University.

Lynn V. Moyes, Research Technician, Dept. of NFS, Utah State University

Richard Merrill, Graduate Assistant, Dept. of NFS, Utah State University.

**Funding Sources:** Western Dairy Foods Research Center

**Objectives:**

1. Strain isolation work will continue to be a primary focus. Many isolates that appear to have reduced Prt activity have been isolated from parent strains of L. delbruecki ssp. bulgaricus and S. salivarius ssp. thermophilus. These will be characterized for peptidase activity. Strains of L. helveticus and Prt- variants of Lactococcus lactis ssp. cremoris will also be evaluated for their potential use in Mozzarella manufacture.
2. Cheese curd will continue to be made from the strains isolated above. In these trials the whey will be tested for protein losses from the curd. A number of rod:cocci culture pairs and ratios will be used to evaluate their effect on the physical properties of the cheese. Measurements of the physical properties of the curd will be used to establish differences among strains, culture pairs, and rod:cocci ratios.
3. The present stretch test will be evaluated to see if it provides the most accurate representation of stretchability.
4. The ability of freezing and Prt- cultures to extend the life of the desired physical properties in Mozzarella cheese during storage will be examined.
5. Direct acid curd and curd from Prt+ and Prt- cultures will be compared at high pH values to evaluate the degree of stretchability and the stability of this property at refrigeration temperatures.

6. Direct acid cheese made with a variety of milk coagulating enzymes will be analyzed to determine the affect of these enzymes on the physical properties of Mozzarella cheese.

## Results:

Experiments were run comparing the helical viscometer and the Instron to measure stretch in Mozzarella cheese. The results were inconclusive.

Six-liter vats of direct acid Mozzarella cheese were manufactured using either chymosin, bovine pepsin, porcine pepsin, or Mucor miehi protease. Four cheeses were made with each enzyme. Stretch using a helical viscometer, melt using a tube test, color using a reflectance colorimeter, moisture, and pH measurements were taken at 1, 7, 14, and 28 d during storage at 4 C. Analysis of variance and correlations were run on all parameters. Cook color was not affected by enzyme type but changed during storage time. Melt increased significantly with time, but was not affected by choice of enzyme. Moisture content of the cheese was not significantly affected by enzyme or by time. As melt increased over time, stretch decreased. The type of milk coagulating enzyme used in the manufacture of direct acid Mozzarella cheese played no role in development of the physical properties.

Presently, Mozzarella cheese is being manufactured using various ratios of rods to cocci and the cheese is being evaluated for stretch, cook color, and melt. This study will be completed in the next two weeks. Mozzarella cheese is also being made with L. helveticus cultures and their affect on changes in physical is currently being measured.

## Impact of Research:

The second leading cheese produced in the U.S. is Mozzarella cheese. Proteinase negative starter cultures used in the production of Cheddar cheese, cottage cheese, and acid casein have been shown to increase yield, along with providing a number of other advantages. If proteinase negative cultures could be used in the production of Mozzarella and other high temperature Italian cheeses, an increase in yield may be possible. Additionally, if the effects of proteinase activity can be measured, there may be a possibility of improvement in the stretching, along with other physical properties. We need to learn more about the effect proteinase activity of the thermolactic cultures have on the physical qualities of these cheese types.

The major purchasers of pizza cheese are concerned that the physical qualities of Mozzarella curd begin to deteriorate at about ten days of age. The proteolytic activity of the starter culture is thought to be the cause of reduction of stretch. Buyers require that the curd be grated, frozen, and stored to maintain the young cheese qualities. By incorporation of less proteolytic thermolactic starter

cultures, we desire to extend these superior qualities. The better and more consistent the physical properties, the more sales for the product.

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- Oberg, C.J., B.C. Weimer, L.V. Moyes, R.J. Brown, and G.H. Richardson. 1990. proteolytic characterization of Lactobacillus delbrueckii spp. bulgaricus strains by amino acid analysis. J. Dairy Sci. (In press).
- Oberg, C.J., A. Wang, L.V. Moyes, R.J. Brown, and G.H. Richardson. 1990. Effect of proteolytic activity of thermolactic cultures on physical properties of Mozzarella cheese. J. Dairy Sci. (In press).
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- Merrill, R., C.J. Oberg, L.V. Moyes, R.J. Brown, and G.H. Richardson. 1990. Effect of Lactobacillus helveticus cultures on physical properties of Mozzarella cheese. J. Dairy Sci. (in progress).

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- Oberg, C.J., A. Wang, L.V. Moyes, and G.H. Richardson. 1989. Effect of proteolytic activity of thermolactic cultures on physical properties of Mozzarella cheese. J. Dairy Sci. 72 (Suppl. 1): (Abstr.).
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**Project Title:** Improved Control of Cheese Manufacture Through Vat Monitoring.

**Personnel:** G. H. Richardson, Professor, Nutrition and Food Sciences, Utah State University

Donald J. McMahon, Professor, Nutrition and Food Sciences, Utah State University,

Remy Grappin, INRA, France;

Michael J. LeFevre, Research Assistant, Nutrition and Food Sciences, Utah State University.

**Funding Sources:** Western Dairy Foods Research Center  
Utah Agricultural Experiment Station.  
Snow Brand Milk Products Co. Ltd., Japan  
Stoelting, Inc., Kiel, Wisconsin  
Stoelting Co. Inc., WI  
Golden Cheese Co., Corona, CA  
Western General Dairies, Twin Falls, ID  
GHR Corp., Logan

#### Objectives:

1. Evaluate the control of curd strength during cottage cheese manufacture. Establish software that would be most helpful for the cheese industry.
2. Use the same system to monitor the coagulation of milk for Cheddar cheese manufacture. Determine the limits of curd strength that would cause significant product loss or prevent sufficient moisture removal. These data would then be applied to expert systems .
3. Determine abilities of chymosin, calcium salts, and lactic cultures in milk for Cheddar cheese to overcome the inability of milk from cows in late lactation to coagulate.
4. Compare the performance of proteinase negative lactic cultures when monitored by the system. Determine if they can perform at constant high cooking temperatures and if they confirm the observations of Linklater and Hall that culture volume is more important than temperature in pH control.

#### Results:

A hot wire coagulation probe was obtained from Snow Brand Milk Products Co., Ltd.. Auxiliary equipment including data acquisition material, power supplies, and pH and temperature sensors were also acquired. Control and measurement of the hot wire was adapted for use with IBM PC/XT/AT computer systems. Software was written to collect data during Cheddar cheese and cottage cheese manufacture. We have shown that the hot wire system will sense agitation and curd healing as well as coagulation of milk in the vat. Tests comparing the coagulation points of milk using a broad range of instrument types are in progress and will help to prepare correlation



values. Information obtained from these other instruments may also be incorporated into the software parameters.

### **Impact of Research:**

The data generated from such an instrument can be useful to provide improved control to every cheese vat. A curd cut time, based coagulation, pH and temperature could decrease losses and improve cheese yields and quality. The ability to measure the length of the heat time and the rates of change of pH and temperature would also benefit the cheese manufacturer. Software programs could be included that would provide more management guidance. Significant savings to the industry could result when enzyme coagulant and other additive costs can be reduced by fine tuning the process through continuous monitoring of the milk in the cheese vat.

**Project Title:** Improved Control of Cheese Manufacture Through Vat Monitoring.

**Personnel:** G. H. Richardson, Professor, Nutrition and Food Sciences, Utah State University;  
Don McMahon, Professor, Nutrition and Food Sciences, Utah State University;  
Snow Brand Milk Products Co. Ltd., Japan;  
Remy Grappin, INRA, France;  
Stoelting Co. Inc., WI;  
Golden Cheese Co., Corona, CA;  
Western General Dairies, Twin Falls, ID;  
GHR Corp., Logan;  
Michael J. LeFevre, Research Assistant, Nutrition and Food Sciences, Utah State University.

**Funding Sources:** Western Dairy Foods Research Center  
Utah Agricultural Experimental Station.

**Objectives:**

1. Evaluate the control of curd strength during cottage cheese manufacture. Establish software that would be most helpful for the cheese industry.
2. Use the same system to monitor the coagulation of milk for Cheddar cheese manufacture. Determine acceptable limits of curd strength that would prevent the development of high moisture cheese due to late cutting or product losses due to early cutting of the curd. These data would then be applied to expert systems.
3. Determine abilities of chymosin, calcium salts, and lactic cultures in milk for Cheddar cheese to overcome the inability of milk from cows in late lactation to coagulate.
4. Compare the performance of proteinase negative lactic cultures when monitored by the system. Determine if they can perform at constant high

cooking temperatures and if they confirm the observations of Linklater and Hall that culture volume is more important than temperature in pH control.

### **Impact of Research:**

The data generated from such an instrument can be useful to provide improved control to every cheese vat. A curd cut time, based coagulation, pH and temperature could decrease losses and improve cheese yield and quality. The ability to measure the length of heat time and the rates of change of pH and temperature would also benefit the cheese manufacturer. Software programs could be included that would provide more management guidance. Significant savings to the industry could result when enzyme coagulant and other additive costs can be reduced by fine tuning the process through continuous monitoring of the milk in the cheese vat.

### **Results:**

A hot wire coagulation probe (Snow Brand, Ltd., Japan) was configured with pH and temperature sensors. Milk substrate coagulation was monitored with this system and four other methods (Formagraph™, Sommer Matsen apparatus, Brookfield™ LVT viscometer, and an Omnispec™). The coagulation time of the hot wire probe was measured at maxima of the first and second derivatives. Coagulation times were compared using three repetitions with three levels of chymosin. The ability of each instrument to detect coagulation time varied from first to last: hot wire probe (2nd der. max.), Omnispec, hot wire probe(1st der. max.), Sommer Matsen, Formagraph and viscometer. The coagulation time measured by the Sommer Matsen method differed significantly from that measured by the hot wire probe (2nd der. max.), Omnispec and the viscometer  $p < .05$ . The hot wire probe system was also used to monitor cheese manufacture in 300 kg batches. Temperature, pH, coagulation, cutting, healing, and stirring could all be detected with the monitor system.

### **Abstract:**

Monitoring cheese manufacture using a hot wire probe. M. J. LeFevre\* and G. H. Richardson. Utah State University, Logan, UT.

**Project Title:** Improved Control of Cheese Manufacture Through Vat Monitoring

**Personnel:** G. H. Richardson, Professor, Dept. of Nutrition and Food Sciences, Utah State University

Don McMahan, Professor, Dept. of Nutrition and Food Sciences, Utah State University

Michael J. LeFevre, Research Assistant, Dept. of Nutrition and Food Sciences, Utah State University.

**Funding :** Western Dairy Foods Research Center and Utah Agricultural Experimental Station.

**Objectives:**

1. Determine the ability of the hot-wire system to detect differences in coagulation time and curd strength. Compare these measurements with other coagulation instruments. These data would aid in preventing high moisture cheese due to late cutting or product losses due to early cutting of the curd.

2. Determine abilities of chymosin, calcium salts, and lactic cultures in milk for Cheddar cheese to overcome the inability of milk from cows in late lactation to coagulate.

3. Use the same system to monitor the coagulation of milk for cottage cheese manufacture. Establish software that would be most helpful for the cheese industry.

**Results:**

ADSA abstract 1991

**Use of hot-wire viscometric measurements to predict physical curd firmness of renneted milk as a function of milk composition.** M.J. LeFevre\*, G.H. Richardson and D.J. McMahon, Utah State University, Logan

Curd firmness was predicted by viscometric measurements using the hot-wire method for renneted milk of various composition. A 2x5 factorial design was set up using 2 levels each of protein, fat, Ca, pH and chymosin. The protein content of skim milk was adjusted by ultra-filtration to 3.25% and 2.75% and the pH was adjusted to 6.54 and 6.28 using lactic acid. Fat and Ca were adjusted to a zero level (no addition) and to 3.25% and .01% respectively by the addition

of cream and  $\text{CaCl}_2$ . Rennet coagulation tests (.03 RU/ml and .015 RU/ml) were run simultaneously using the hot probe instrument, with pH and temperature sensors, and a Formagraph. The analysis of variance indicated that all 5 factors caused a significant ( $p < .05$ ) effect on the time required to reach the  $K_{20}$  value (approximate cut-time for renneted milks) on the Formagraph. Significant interactions included chymosin x pH, chymosin x protein, and fat x protein ( $p < .05$ ). Coagulation time, maximum first derivative value and selected area values of the first derivative curve of the hot probe data were used along with milk composition variables to predict the Formagraph  $K_{20}$  value using step-wise regressions. Linear correlation coefficients ( $R^2$ ) ranging from .90 to .96 were obtained in predicting the time from chymosin addition to the  $K_{20}$  point.

Other tests have also been completed that demonstrate the effect of changing milk temperature on the hot-wire curve. Poor coagulating milk has been evaluated by the system and tests are presently underway using late lactation milk.

### Impact of Research:

The data generated from such an instrument can be useful to provide improved control to every cheese vat. A curd cut time, based coagulation, pH and temperature could decrease losses and improve cheese yield and quality. The ability to measure the length of heat time and the rates of change of pH and temperature would also benefit the cheese manufacturer. Software programs could be included that would provide more management guidance. Significant savings to the industry could result when enzyme coagulant and other additive costs can be reduced by fine tuning the process through continuous monitoring of the milk in the cheese vat.

### Publications

LeFevre, M.J. and Richardson, G.H. 1990. Monitoring cheese manufacture using a hot wire probe. J. Dairy Sci. 73 (suppl. 1):74

Project Outline  
UTAH AGRICULTURAL EXPERIMENT STATION

Project No. \_\_\_\_\_

Fund: \_\_\_\_\_

Title: Utilization of dairy-derived polysaccharides in food and nonfood systems

Personnel: Robert L. Olsen, Ph.D.  
Gour Choudhury, Ph.D.  
Conly L. Hansen, Ph.D.

Original Starting Date:

Date of Last Revision:

Duration of Current Project:    July 1, 1987                      June 30, 1989  
   Starting Date                      Estimated Completion Date

Institutional Units Involved:    Department of Nutrition & Food Sciences

Authorized:

<hr/>	<hr/>	<hr/>	<hr/>
Project Leader	Date	Co-Project Leader	Date

<hr/>	<hr/>	<hr/>	<hr/>
Department Head	Date	Co-Project Leader	Date

<hr/>	<hr/>
Dean	Date

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Director, Agr. Exp. Station	Date

## JUSTIFICATION

The ability of dairy farmers to produce milk has exceeded the market demand resulting in greater government purchases. By using milk components as raw materials in the manufacture of food ingredients and nonfood chemical stocks, the surplus milk problem could be reduced.

Industrial by-products are receiving considerable attention since they represent marginal income from fixed-cost waste products, and they lower the BOD of factory effluent, if consumed. Utilization of excess cheese whey currently remains a problem with only 63% of the 37 billion kg of whey produced annually in the United States being marketed (Nolan, 1983). Successful utilization of whey as a fermentation medium for exopolysaccharides could expand that market.

As the practice of ultrafiltration increases, profitable utilization of permeates from whey and milk will become more critical. Fermentation of permeate to produce useful polysaccharide material could represent a viable outlet for that by-product.

## PREVIOUS WORK AND PRESENT OUTLOOK

A common feature of microorganisms is their ability to produce polysaccharides. Polysaccharides are located within the cell, as part of the cytoplasmic membrane, in the cell wall and outside the cell wall as exocellular polysaccharides. Several organisms such as Xanthomonas campestris and Leuconostoc mesenteroides can produce gums by using glucose, sucrose, or lactose and a suitable nitrogen source as nutrients (Stauffer and Leeder, 1978). Sheelhaass (1983) examined the exopolysaccharides produced by several lactic acid bacteria. Schwartz and Bodie (1984) reported production of viscous dextran-containing whey broths using

Leuconostoc mesenteroides. Several patents have been issued covering fermentation of whey broths using Leuconostoc mesenteroides and Xanthomonas campestris to produce a thickening polymer (Schwartz and Bodi, 1984A, 1984B, 1984C). The patents by these workers do not cover fermentations from lactic acid bacteria nor have they reported product application information except for limited viscosity data.

Sarkar et al. (1986) reported an osmophilic yeast (Moniliella pollinis) that can produce an extracellular, highly viscous polysaccharide in a simple mineral medium. The polysaccharide produced was white, gum-like and highly soluble in cold and hot water.

Factors affecting exopolysaccharide production have been studied. Sutherland (1977) stated that the involvement of an isoprenoid carrier is one of the most important factors affecting exopolysaccharide synthesis. Frequently, exopolysaccharide production is greater at lower growth temperatures since less isoprenoid phosphate is required for cell wall synthesis. Scheelhaass (1983) suggested incubation at a low temperature (25°C for Streptococcus cremoris), maintenance of a relatively high pH (6.2), growth when nutritive constituents are lacking, and less frequent transfer of cultures to maximize exopolysaccharide synthesis.

Enzyme studies in connection with the capsule- and slime-forming capacity of lactic streptococci have been accomplished using p-fluorophenylalanine (Forsen and Veli-Mies, 1981). Forsen et al. (1979) have also examined differences between slime-forming lactic streptococci using polyacrylamide gel electrophoresis of soluble cell proteins.

While lactic acid bacteria polysaccharides contribute to the infrequent defect of ropy milk, they also function beneficially to stabilize and thicken the body of yogurt (Meiklejohn, 1977). A specific application of



pure exopolysaccharides as stabilizers in frozen yogurt has been examined (Scheelhaass, 1983). Although microbial exopolysaccharide production in whey-based media has been reported, with the exception of the limited work on yogurt, information on their functional characteristics in food products is lacking.

In the coagulation of milk, enzymes function by splitting the Phe-Met bond of k-casein. Exopolysaccharides produced by lactic acid bacteria in the starter culture may affect this reaction and also the rate of flocculation of paracasein micelles. Membrane-derived lipoteichoic acid has recently been found on the surface of slime-forming Streptococcus cremoris cells and it was suggested that this lipoteichoic acid may cause problems during detachment of capsular material by forming micelles with proteins and carbohydrates (Kontusaari et al. 1985).

Polysaccharides are used in many foods to impart certain functional characteristics. These include adhesion, binding, crystal inhibition, clarification, coating, emulsification, encapsulation, film formation, foam stabilization, gelation, stabilization and thickening. Formation of polysaccharides from microbial activity in whey-based media has been demonstrated. For this alternative use of surplus whey to find practical application, information will be required comparing these polysaccharides to those currently on the market.

Although polysaccharide gums are used in a large variety of food products, the total quantities used for food purposes are small compared to the nonfood industrial applications. Paper and textile industries, representing a much larger market, use gums for coating and sizing agents. Other major industrial markets of polysaccharides include paint, pesticides, fertilizers, feed supplements, metal working, explosives and hydrolubes.

Adhesives, ceramics, cleaners, detergents, fire retardants, ink, mining, photography, polymerization, deodorants, and lithography represent smaller industrial markets.

Information from these studies both in food and nonfood applications could develop substantial new markets for milk and milk by-products.

Preliminary investigations in this laboratory have involved acquisition of equipment, establishment of test and analytical procedures, and collection and isolation of polysaccharide-producing strains from various sources. Optimal media characteristics using whey and milk are being examined.

#### OBJECTIVE

The objectives of this project are:

1. Examine the production of exopolysaccharides by lactic acid bacteria in whey based and milk based media using pH control.
2. Examine the suitability of whey or whey permeate as a nutrient media for the osmophilic yeast, Moniliella pollinis, to produce extracellular polysaccharide.
3. Examine energy and cost data to determine the economic feasibility.
4. Examine functional properties of the polysaccharides in several food applications in comparison to gums in current use.
5. Investigate the use of dairy-derived polysaccharides in paper, textile and other nonfood applications.
6. Examine the protein quality of the bio-mass.

## PROCEDURES

### Strain Isolation

Exopolysaccharide producing strains will be isolated from Streptococcus cremoris, Streptococcus thermophilis, Streptococcus lactic, and Lactobacillus bulgaricus cultures by inoculating into sterile 11% reconstituted non-fat dry milk (RNFD) followed by plating on Elliker agar spread plates. Incubation will take place in an anaerobic chamber using disposable CO<sub>2</sub>/H<sub>2</sub> gas packs (BBL, Cockeysville, MD). Colonies will be examined with a sterile inoculation loop touched to the surface. Sticky colonies will be reinoculated into RNFD. Ropy cultures will be plated a second time (Scheelhaass, 1983).

### Fermentation Studies

Media will be prepared from several concentrations of reconstituted sweet dairy whey, with and without added carbohydrate, yeast extract, and K<sub>2</sub>HPO<sub>4</sub>. Following sterilization and inoculation, fermentation will take place in a Microferm Fermenter (New Brunswick Scientific). Temperature will vary from 25 to 35 C according to the type of culture inoculated. The pH will be maintained at 6.2 with NH<sub>3</sub> injection. The anaerobic fermentation will use no gas sparging (Schwartz and Bodie, 1984).

Using previously determined optimal parameters, batches will be freeze dried for further studies. Both crude and purified material will be examined.

### Yeast growth and polysaccharide production

A preculture will be prepared by inoculating a media containing 10% dextrose, 1% yeast extract and 0.1% urea with the mycelium of Moniliella pollinis in laboratory shaken flasks at 27°C for 48 h. This preculture will

then be used to inoculate whey or whey permeate in laboratory shaken flasks and will be incubated at 27°C. The growth of the culture will be monitored by centrifuging the mycelium from the culture media at regular time intervals and estimating the dry weight of the well-washed cell mass.

### Chemical Composition

Carbohydrate composition will be estimated on all fermentates by the phenol sulfuric acid reaction (Dubois et al, 1956). Classes of carbohydrate will be estimated by the method of Dische (1962). Reducing carbohydrates will be examined by the Munson-Walker method (AOAC, 1980). Purified carbohydrate will be examined by hydrolysis and HPLC analysis (Richmond et al, 1981).

### Quality of Cell Mass

Harvested cell mass will be analyzed for protein content by the Kjeldahl method. Amino acid analysis using HPLC will be run on composite samples to determine protein quality. Nutritional quality of the biomass will be evaluated by feeding experiments.

### Evaluation

Basic properties of the fermentates will be examined. Density measurement will be by mass weight. Viscosity will be measured using a Brookfield Viscometer (Brookfield Engineering Laboratories, Inc., Stoughton, Massachusetts). Solubility will be measured according to the method of Ewart and Chapman (1952) and gel strength according to the IFT method (IFT 1959).

Functional properties of the fermentates will be examined in model food

systems in comparison to currently used polysaccharides. The food systems are listed in Table 1. Thickening, whipping, and stabilizing abilities will be emphasized.

Table 1. Food Systems

Function	System	Standard	Test
Binding agent	Sausage	Gelatin	Instron, UTM
Crystallization inhibitor	Ice cream	Carrageenan	Sensory evaluation
Emulsifier	Salad dressing	Xanthan	Visual examination
Foam stabilizer	Whipped topping	CMC	Funner-cylinder
Gelling agent	Jam	Pectin	IFT method
Stabilizer	Chocolate milk	Carrageenan	Centrifugation
Syneresis inhibitor	Cheese	Alginate	Visual observation
Thickening agent	Pudding	Starch	Brookfield viscometer
Whipping agent	Whipped topping	Carrageenan	Initial volume

Protective effects on whey proteins during heating will be examined using polyacrylamide gel electrophoresis. The spray-dried fermentate cultured with exopolysaccharide producing bacteria will be compared to an uncultured whey powder.

Functional differences will be observed in a bakery product system. Evaluation will be performed using an Instron Universal testing machine.

This project will provide an opportunity to examine the effects of polysaccharide material on rennet and acid coagulation. Effects on rennet activity will be observed by soluble N measurement. Coagulation characteristics will be examined with a CTM-1000 Curd Tension Monitor (CEM Corporation, Indian Trail, North Carolina). Some degree of association between proteins and carbohydrates is expected and this type of information may be useful in efforts to develop new dairy products.

FINANCIAL SUPPORT

	<u>Year 1</u>	<u>Year 2</u>	<u>Total</u>
Salaries			
Post Doctoral Associate (1/2 time)	11,000	11,770	22,770
Lab helper	4,000	4,000	8,000
Graduate Assistant	7,200	7,200	14,400
Fringe Benefits	4,440	4,683	9,123
Supplies	4,000	4,000	8,000
Equipment	5,900	4,800	10,700
Travel	1,250	1,700	2,950
Publication costs	<u>300</u>	<u>300</u>	<u>600</u>
Total	<u>38,090</u>	<u>38,453</u>	<u>76,543</u>

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## TITLE:

Evaluation of Milk Protein and Microbial Polysaccharide Interaction

## PERSONNEL:

R. Olsen  
C. Hansen  
G. Choudhury

## FUNDING SOURCES:

Western Dairy Foods Research Center

## OBJECTIVES:

- 1) To determine effects of exopolysaccharide on renneted milk gel syneresis
- 2) To determine effects of exopolysaccharide on cheese quality and nutrient recovery.

## RESULTS:

1. Ropy strains of Lactococcus Lactis decreased the rate of whey syneresis in renneted milk gels. Various methods were investigated including drainage of cut curd in a cheese-cloth lined funnel, UV absorption of diluted whey, centrifugation and periodic drainage of coagulated curd. Most of the results reported were obtained using the periodic drainage method. Comparisons were made to commercially available polysaccharides commonly used in dairy products (see graph 1). Effects of pH, heat, culture level, total solids and fat were examined. Ropy cultures generally reduced syneresis. The effect was more pronounced at higher pH values (see graphs 2-5).
2. Lab scale trials were performed in 4 l stainless steel containers with temperature controlled water baths. Fresh cultures were prepared from ropy and non-ropy strains of Streptococcus thermophilus.

Fourteen trials were completed and the final cheese were analyzed for total solids, protein, fat and ash. Neither the unadjusted nor the adjusted yields were significantly different. The nutrient recovery data also shows no significant difference.

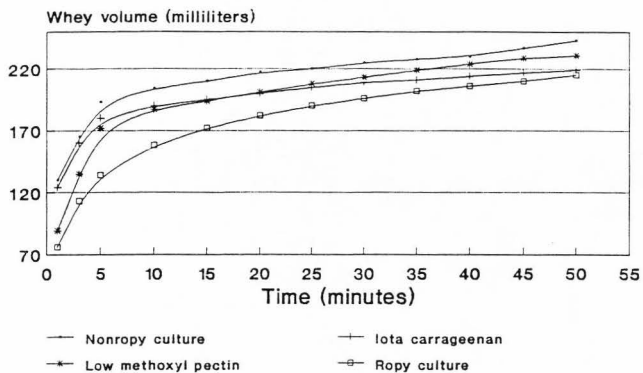
Although the adjusted yields and nutrient recoveries were not significantly different, it is possible that interactions were too subtle to be observed. One fact may have been the amount of polysaccharide present. The amount of starter culture used was based upon common usage levels. Since polysaccharide yield in the culture was approximately 5g polysaccharide/1000g culture, the amount of polysaccharide was diluted to 0.005% in the cheese-milk.

In order to increase the amount of polysaccharide added to the cheese-milk without increasing the amount of Streptococcus thermophilus and to avoid the errors associated with small scale batches, a large pilot scale trial was carried out. Ropy and non-ropy Lactococcus Lactis cultures were used to increase the amount of polysaccharide. High cooking temperatures controlled subsequent acid production from the more temperature sensitive L. Lactis culture. Yields, again, were not significantly different (see charts 1 and 2). The ropy vat did show improved protein recovery, but not enough to cause a significant increase in yield.

### ECONOMIC EFFECTS:

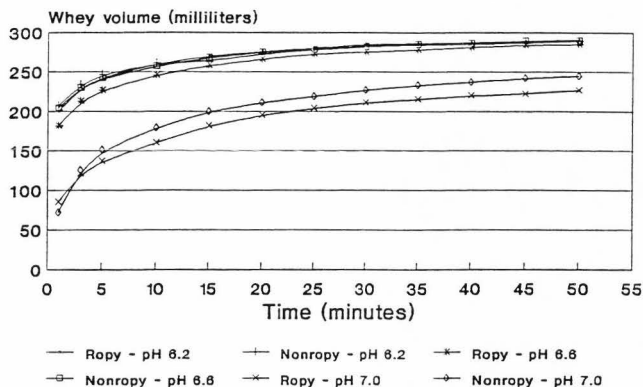
1. Potential method for moisture control in milk gels which have experienced various treatments affecting water holding capacity.
2. Incorporation of polysaccharide material into the cheese curd may improve body and texture. At this point, it is not apparent that polysaccharide incorporation has been accomplished. Body and texture effects are being evaluated.

Figure 1. Effects of polysaccharide materials on syneresis with ropy and nonropy cultures



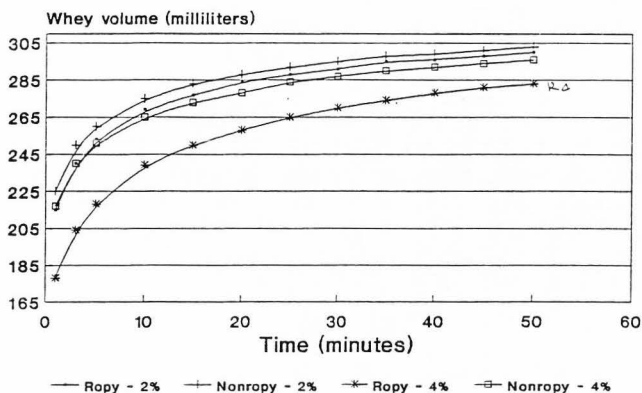
Graph 1

# Effects of pH on syneresis using ropy and nonropy cultures



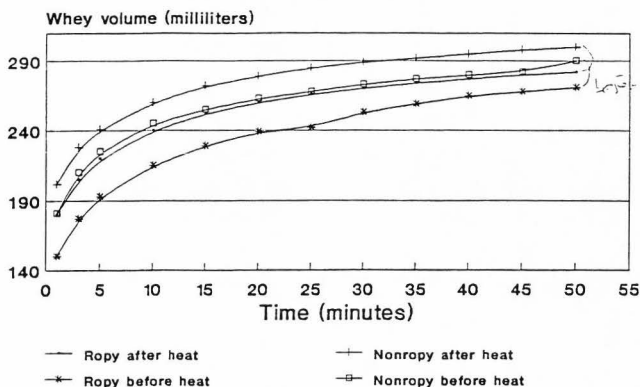
Graph 2

# Effects of culture level on syneresis using ropy and nonropy cultures



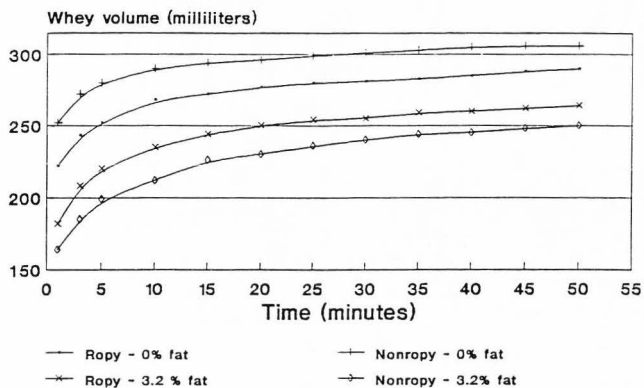
Graph 3

# Effects of heat on syneresis using ropy and nonropy cultures



Graph 4

# Effects of fat on syneresis using ropy and nonropy cultures



Graph 5

CHEESE YIELD DATA (LARGE TRIAL)

	<u>ROPY</u>	<u>NON-ROPY</u>
Unadjusted Yield	7.7	7.7
Adjusted Yield (45% H <sub>2</sub> O)	8.3	8.3

Chart 1



**Project Title:** Causes and Prevention of Sticky Texture in Mozzarella Cheese

**Personnel:** Gary H. Richardson, Dept. of Nutrition and Food Sciences, Utah State University.

Richard K. Merrill, Dept. of NFS, USU

**Funding:** Western Dairy Foods Research Center

**Objectives:**

1. Survey local manufacturers of Mozzarella cheese to obtain data concerning prevalence and present measures used to deal with the problem.
2. Obtain commercial samples of Mozzarella cheese that exhibit stickiness properties and analyze their physical and microbiological properties. Properties to be examined include moisture, fat content, protein content, pH, manufacturing profile, salt concentration, microbial count, culture type and strain used, and calcium content.
3. Assess the affects of freezing temperature, storage time and temperature, shredding, and thaw time and temperature on stretch, melt and cook color of low moisture part skim Mozzarella cheese.

**Results:**

Data gathered to date supports the findings of Masi and Addeo (1986). They observed that cheese becomes softer and more difficult to shred with increasing FDB and moisture content. We have shown that freezing, storing, and shredding significantly affect the stretch and melt of Mozzarella cheese, but do not affect cook color. Also, storing, thawing, and shredding parameters have been established so as to obtain optimal stretch and melt in frozen Mozzarella cheese. Further data is being collected to see if manufacturing parameters, culture type, or the strain used may play a role in the development of sticky texture in Mozzarella cheese.

Masi, P., and F. Addeo. 1986. Some practical problems and their solution in the manufacture of Mozzarella cheese. Proc. 5th Annu. Marshall Invit. Ital. Cheese Sem., Madison, WI.

**Impact of Research:**

Knowledge of manufacturing and storage practices that lead to poor product quality will allow Mozzarella cheese producers to manufacture better quality product. However, manufacturers must balance stretch and melt, because freezing and storing frozen affect stretch and melt oppositely.

**Publications:**

Oberg, C. J., Merrill, R. K., Brown, R. J., and Richardson, G. H. 1991. Effects of freezing, thawing, and shredding on low moisture part skim Mozzarella cheese. (submitted to J.Dairy Sci.).

# Ultra-High Temperature Processing

## **Controlling age gelation of UHT sterilized milk concentrates**

**Annual Report Date:** 30 June 91    **Project Term:** 1 Jul 90 — 30 Jun 92

### **Personnel**

Principal Investigator: Dr. D.J. McMahon

Graduate Student:

**Funding Sources:** Western Dairy Foods Research Center  
USDA Agricultural Research Service

### **Objectives:**

Age gelation of ultra-high temperature (UHT) milk concentrates has hindered the commercial use of milk concentration as a means of lowering transport costs. There has been some work conducted on developing stable UHT milk concentrates but at best, an empirical approach is taken to extend shelf life. Composition of milk, severity of heat treatment, sequence of operation, homogenization, use of additives such as polyphosphates and sucrose, total solids, and enzyme treatment have all been shown to affect age gelation (and hence shelf-life) of UHT milk concentrates.

When milk is concentrated, its rheological and heat transfer characteristics are altered. Heat sterilization also affects these properties. To optimize the UHT processing of milk concentrates it is necessary to know how milk behaves throughout the UHT temperature profile. The effect of compositional changes and process parameters on age gelation will be studied in this project. By knowing how these parameters affect milk, it will then be possible to systematically develop a process for UHT sterilization of milk concentrates that can be successfully stored at ambient temperatures.

The specific objective of this project is to solve the age gelation problem by:

1. Determining effects of the process parameters of UHT heating on age gelation of milk concentrates.
2. Determining influence of compositional changes of milk concentrates on age gelation after UHT processing.
3. Using the data collected above to determine optimum conditions for UHT processing of milk concentrates.

### **Results:**

It was anticipated that this work would commence in 1990 but because of research commitments on our UHT equipment it was not possible to start another graduate student on this project. Now that some of the other UHT projects in our department are coming to conclusion there is now time available.

**Impact of Research:**

The overwhelming success of dairy production in the United States has created a situation that calls for more attention to milk utilization and marketing. Development of new dairy products has taken precedence over production of more milk. At the same time, we are becoming increasingly aware that a global approach must be taken in the marketing of our dairy products. Two marketing strategies that are of importance are EXPORT and MILITARY. In both cases, transportation costs are of very high. By reducing the bulk of a product, such as by concentrating milk, transportation costs can be reduced and furthermore, for many military applications it would also alleviate space storage limitations of supplying "fresh" milk to military personnel.

International markets for U.S. dairy products could be developed if attention could be directed to manufacture of stable products from our surplus dairy production. The competitive position of the U.S. would be enhanced by new and better quality products and our surpluses of dairy commodities would be reduced. Production of dairy products other than powdered milk, butter and cheese with long shelf lives should be a major priority.

Specifically, for this project, a way of producing rehydratable milk concentrates that can be stored at ambient temperatures is to be developed. This is needed to make U.S. dairy products more widely available on the world market. The major limitation to production of such a product is the irreversible gelation of UHT sterile milk concentrates that occurs after exposure to higher-than-refrigerator temperatures over a long period of time. The gelling phenomena must be understood and means devised to prevent gel formation before rehydratable milk concentrates made from surplus U.S. milk can be sold abroad.

An additional benefit of this project is that it would provide information on extended shelf life of dairy products. This would be useful to dairy processors who are looking at entering the expanding food service business, and in which extended shelf life is of importance.

**Publications and Completed Theses:**

None

**Project Title:** Function of whey proteins and lactose in age gelation of ultra-high temperature sterilized milk concentrate

**Personnel:** Donald J. McMahon, Principal Investigator, Dept. of Nutrition and Food Sciences, Utah State University.

Mrudula Kalpalathika, Research Associate, Dept. of NFS, USU.

Venkatachalam Narayanaswamy, Graduate Student, Dept. of NFS, USU.

Bashir Yousif, Graduate Student, Dept. of NFS, USU.

Kevin Sorensen, Laboratory Technician, Dept. of NFS, USU.

**Funding Sources:** Western Dairy Foods Research Center  
USDA Agricultural Research Service  
Utah Agricultural Experiment Station

### **Objectives:**

The mechanism by which age gelation in UHT sterilized milk concentrates occurs is still unknown. There have been many factors implicated and at best an empirical approach is taken to extend shelf life of sterilized milk products.

UHT sterilization promotes association between  $\kappa$ -casein and  $\beta$ -lactoglobulin. In milk concentrates the concentration of whey proteins and lactose are increased. Their role in the age gelation process will be studied in this project. The specific objectives of this project are to:

1. Determine the fate of  $\beta$ -lactoglobulin during storage of UHT sterilized milk concentrates.
2. Determine the influence of lactose concentration of milk concentrates on age gelation.
3. Monitor changes in casein micelle structure during storage of UHT sterilized milk concentrates.

### **Results:**

#### Objective 1: $\beta$ -lactoglobulin.

The use of  $^{14}\text{C}$ -labelled  $\beta$ -lactoglobulin in UHT milk experiments has required that a laboratory scale UHT system be developed so that contaminated equipment can be properly handled. Our commercial pilot-scale Alfa-Laval UHT system

cannot be used for this work as its contamination would render it unusable for other work. The laboratory UHT processor (built using 9 mm SS tubing) was designed to duplicate the Alfa-Laval system with pre-heat treatment to 72°C over 60 s followed by indirect heating to 140°C over 90 s. The milk is held at UHT temperatures for 4 s and then cooled to less than 30°C. No homogenization is required as skim milk is used in these trials.

Preliminary trials have been conducted to determine that an equivalent amount of protein denaturation occurs in the two systems. After heat treatment the samples are filled into sterile containers in a cabinet with a positive flow of filtered air to prevent bacterial contamination.

## Objective 2: Lactose

### Adjustment of Lactose Levels in Milk

Preliminary trials have been conducted to determine the best procedure for removing lactose from milk followed by addition of lactose and sucrose at specified levels. Ultrafiltration followed by repeated diafiltration was used to remove most of the lactose from milk. The efficacy of diafiltration was determined by ultrafiltering 50 gallons of skim milk in our 5 m<sup>2</sup> spiral UF membrane system using the following treatment scheme. All milk concentrations are expressed on a volume reduction basis.

Skim milk (1X)
Ø
UF Concentration to 5X
Ø
Dilute to 1X with Water
Ø
UF Concentration to 5X
Ø
Dilute to 1X with Water
Ø
UF Concentration to 5X
Ø
Dilute to 1X with Water
Ø
UF Concentration to 3X

After this treatment the lactose levels were measured using an enzymatic method and by using the three diafiltration treatments we reduced lactose content from 5.1% in the original skim milk to 0.032% in the final 3X skim milk concentrate.

Our original plan was to add lactose at levels of 2.5 g, 5.0 g and 7.5 g per 100 ml skim milk concentrate. This was done and total solids and protein content were measured using microwave oven and Kjeldahl methods respectively as shown in the following table:

Sample type	Protein(%)	Total solids(%)
Skim milk (1X)	3.27	9.48
3X DF SM	9.67	13.4
3X DF SM(+2.5%)	9.23	15.5
3X DF SM(+5.0%)	9.19	17.4
3X DF SM(+7.5%)	8.91	19.6

It was found however, that when the sample with 7.5% added lactose was refrigerated, some of the lactose crystallized even though this does not occur in milk concentrates made using reverse osmosis that have lactose levels of 10% or more. This crystallization of lactose was presumably due to lactose powder being primarily  $\alpha$ -lactose (which is less soluble than  $\beta$ -lactose) while the naturally occurring ratio in milk is of the order of 60%  $\beta$  : 40%  $\alpha$ -lactose. With the need to refrigerate the milk concentrate there is insufficient time to allow mutarotation of  $\alpha$ -lactose to  $\beta$ -lactose before crystallization occurs. For subsequent trials the levels of lactose added were set at 3 and 6%.

In the planned experimental trials, a simulated milk ultrafiltrate salt solution (Jenness-Koops buffer) will be used as the diafiltrate liquor rather than water so that the salt balance of milk can be maintained. It was observed however that when this salt solution was heated to 50°C (the ultrafiltration temperature) there is an irreversible precipitation of calcium (magnesium) phosphate. This occurs because the calcium phosphate becomes less soluble as temperature is increased and although this also happens in milk there is a natural protective affect provided by the casein micelles.

This problem was overcome by reducing the calcium content of the buffer by 30% and adding it cold to the concentrated milk. The diluted milk could then be re-heated to 50°C without precipitation occurring.

#### Measurement of Lactose-Protein Interactions

In order to follow the extent of lactose-protein interactions that occur because of Maillard browning reactions it is necessary to have a method to measure the available lysine present on the proteins of UHT milk.

A common method to measure available lysine is to react 2,4 dinitrofluorobenzene (DNFB) with free  $\epsilon$ -amino groups of lysine followed by acid hydrolysis to convert the proteins to individual amino acids and their dinitrophenyl (DNP) derivatives. The resultant hydrolysate is then treated and ether extracted to remove interferences from DNP-arginine and DNP-derivatives of the N-terminal amino acids of the milk proteins. Because DNP-lysine is yellow, the available lysine can then determined by measuring absorbance at 435 nm. This was found not to be a suitable method as some component of the milk concentrate (possibly calcium or phosphate salts) interfered with the color development.

We then attempted to determine DNP-lysine using an amino acid analyzer. This also was unsuccessful as DNP-lysine eluted from the ion exchange column at the same retention time as glycine.

Finally, a reversed-phase HPLC procedure using a C-18, 250 x 4.6 mm column was tried and proved successful. The eluant was a mixture of 80% 0.01 M acetate buffer and 20% Acetonitrile and the eluted derivatives were



monitored by measuring absorbance at 436 nm. Retention time of DNP-lysine was well separated by several minutes from dinitrophenol which is one of the main interfering compounds in the extraction method. In this case the derivatives are being separated by their different affinities to the HPLC column rather than by solvent extraction. It has also been reported that a complete separation of DNP-lysine from DNP-arginine occurs. The amount of available lysine can then be determined from a standard curve prepared by using different amounts of DNP-lysine and subjected to the same HPLC conditions.

### Objective 3: Casein Micelle Structure

The microstructure of UHT milk concentrates was investigated using electron microscopy in conjunction with Dr. Miloslav Kalab, Food Research Centre, Agriculture Canada, Ottawa, Ontario, Canada. It was observed that when 3X skim milk concentrate is heated to 140°C for 4 s, about 60% of the whey proteins are denatured and the casein micelles undergo a large increase in size. This size increase is due to complexing of  $\kappa$ -casein with denatured  $\beta$ -lactoglobulin followed by further aggregation of denatured  $\beta$ -lactoglobulin onto the micelle surface.

When such UHT samples are stored and eventually age gel, it was observed that many of the micelles were connected by thin threads of material. The microstructure of such gels was completely different to rennet milk gels. When rennet is added to non-UHT milk the casein micelles collide and form a gel structure in which the micelles are in close contact. However, if UHT milk concentrates (such as 3X UF concentrates) are renneted the gels have less integrity and there is not the same level of intimate contact between micelles. It appears that the layers of  $\beta$ -lactoglobulin on the micelle surface interfere with aggregation. At this stage of our work, however, we are unable to define the composition of the filamentous material between micelles in age-gelled UHT milk.

### **Impact of Research:**

The overwhelming success of dairy production in the United States has created a situation that calls for more attention to milk utilization and marketing. Development of new dairy products has now taken precedence over production of more milk.

International markets for U.S. dairy products could be developed if attention was directed to manufacture of stable products from our surplus dairy production. The competitive position of the U.S. would be enhanced by new and better quality products and our surpluses of dairy commodities would be reduced. Production of dairy products other than powdered milk, butter and cheese with long shelf lives should be a major priority. Specifically, for this project, a way of producing a rehydratable milk concentrate which will not gel before reaching the intended consumers is to be developed. This would make US dairy products more widely available on the world market.

The major limitation to production of such a product is irreversible gelation after storage at higher-than-refrigerator temperatures over a long period of time. The gelling phenomena must be understood and means

## Product Quality

Effect of milk-clotting enzymes and starter bacteria  
on the yield of laboratory scale Cheddar cheese

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Logan, UT 84322-8700

and

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Lexington, KY 40546

## ABSTRACT

The effect of six milk clotting enzymes and three different strains of Streptococcus cremoris on the yield of Cheddar cheese were determined. Two lots of milk with identical protein but different fat contents were used for cheese making with the different enzymes. For each enzyme, three vats of cheese were made from the same milk. Four vats of cheese were made from the same lot of milk for each strain of S. cremoris. Neither milk clotting enzymes nor strain of S. cremoris were different in their effect on the yield of cheese. Milk composition had a significant effect on the yield of cheese.

## INTRODUCTION

Clotting of milk is a very important step in cheese making. Apart from the primary action of milk clotting enzymes on  $\kappa$ -casein, partial proteolysis of casein by these enzymes (10) or starter organisms results in casein losses and subsequent low cheese yields. The effects of various commercial milk clotting enzymes on cheese yield have been compared individually to chymosin (6), the most traditional enzyme used in cheese making. A collective comparison of the effect of more than two enzymes using the same milk and identical manufacturing condition is not available. Such a comparison is difficult because yield studies are often done on pilot or industrial scales and yields are not quantitated accurately. Laboratory scale cheese making is also difficult because of equipment design and potential problems in controlling cutting, cooking and agitation. Hicks et al (5) described equipment for laboratory scale cheese manufacture. This offers a potential for comparing several milk-clotting enzymes and cultures and their effects on cheese.

The purpose of this study was to compare the effects of chymosin, bovine pepsin, porcine pepsin, proteases from Mucor miehei, Mucor pusillus and Endothia parasitica on cheese yields. The effects of Streptococcus cremoris strains MLI, HP and 30/70 on cheese yields were also compared.

## MATERIALS AND METHODS

### Milk

Raw milk was obtained from the University of Kentucky dairy farm and immediately pasteurized (63°C/30 min).

### Enzymes

Ion-exchange purified calf chymosin was obtained from the New Zealand Rennet Cooperative. Lyophilized porcine pepsin was from Sigma Chemical Co. Bovine pepsin was obtained by purification of bovine rennet (commercially called 'bovin'). Bovin was donated by Chr. Hansen's Laboratory, Inc. The bovine rennet was adjusted to pH 5.0 with 1 M HCL and saturated with ammonium sulfate to precipitate chymosin. The supernatant was removed after centrifuging (20,000 xg for 45 min) and dialyzed against .0001 M acetate buffer (pH 4.5). The dialysate which appeared as a single band on polyacrylamide gel electrophoresis (PAGE) was lyophilized before use. Microbial proteases from Mucor miehei and Mucor pusillus were donated by Miles Laboratories Inc. The protease produced by Endothia parasitica was obtained from Pfizer Inc.

Milk clotting activities of all the enzymes (expressed as clotting units per milliliter [CU/ml] ) were tested against standard rennet (Chr. Hansen's Laboratory, Inc.) on the raw milk by the method described by Ernstrom (4).

## Cultures

Cultures of Streptococcus cremoris strain MLI, HP and 30/70 were used. The MLI and HP cultures were donated by Chr. Hansen's Laboratories, Inc. The two strain commercial culture (30/70) was obtained from Miles Laboratories Inc. Each culture was propagated in 10% sterile nonfat dry milk (NDM) with external pH control (9).

Activities of the cultures were tested in the milk before use.

## Cheese Making with Enzymes

Cheddar cheese was manufactured by the traditional 4.25 h (setting to milling pH of 5.4) method (7) with the laboratory equipment described by Hicks et al. (5). Seven kilograms (7.0 kg) pasteurized milk was used in each vat. Identical amounts of annatto cheese color (1 ml) and .7% starter culture (S. cremoris 30/70) were added to each vat. No ripening time was allowed prior to enzyme addition. The enzymes were added at levels necessary to obtain identical cut time of 30 min. Eight-tenths milliliter (.8 ml) chymosin (135 CU/ml), 1.25 g bovine pepsin (90 CU/g), .03 g porcine pepsin (3365 CU/g), .9 ml Mucor miehei protease (115 CU/ml), .8 ml Mucor pusillus protease (135 CU/ml), and .6 ml Endothia parasitica protease (180 CU/ml) was used. Each enzyme was diluted in 25 ml .0001 M HCl before use. A uniform setting temperature of 30°C was used in all vats. Curd was cut 30 min after addition of enzyme and the cut curd was allowed to heal for 15 min. The curd was cooked to 39°C within 30 min and held for 1 h. Dipping was accomplished by pouring curd and whey through a weighed cheese cloth which was used

1  
2 during subsequent drainage of whey during cheddaring and for lining of  
3 cheese hoops. Curd was milled in place at pH 5.4, salted (2.75 g  
4 salt/kg milk) and hooped. The curd was pressed overnight at 170 kPa  
5 in a horizontal hydraulic press. Three vats of cheese were made per  
6 enzymes from each lot of milk. The experiment was repeated for a  
7 second lot of milk.

#### 8 9 Cheese Making with Cultures

10 Four vat lots of Cheddar cheese were made per strain of S.  
11 cremoris (MLI, HP or 30/70) from the same lot of milk. The cultures  
12 were used at levels necessary to produce identical activity such that  
13 the pH at mill (4.25 h after addition of culture to milk) was 5.4.  
14 Porcine pepsin (.03g) was used as the clotting enzyme in each vat.  
15 The rest of the cheese making process was followed as previously  
16 described.

#### 17 18 Sampling

19 Milk samples were taken after pasteurization, directly from the  
20 bulk tank. Whey samples were drawn from 2.54 cm below the surface of  
21 the whey in the vat with a pipette before draining. Cheese samples  
22 were taken from the corners and center of cheese blocks immediately  
23 after pressing (8).

#### 24 25 Compositional Analysis

26 Fat in milk and whey samples were determined by the Mojonnier  
27 modification of the Rosse-Gottlieb method (1). Cheese fat was by the  
28



Babcock procedure (1). Total nitrogen in milk, whey and cheese samples were by the Kjeldahl method (2). A nitrogen conversion factor of 6.38 was used in converting nitrogen to protein. Cheese moisture was determined by measuring weight loss after drying 2-3 g cheese in a forced-air oven at 110°C for 16 h (8).

### Statistical Analysis

For cheese made with the different enzymes, a two factor (enzyme and milk) analysis of variance (3) was done for each variable. A one-way completely randomized analysis of variance (3) was used to show the effects of strain of starter culture on yield.

## RESULTS

### Composition of Cheese and Whey

Percent fat, protein and moisture in the cheese made from the same lot of milk but with different enzymes were not significantly different (Table 1). The percent fat and protein in the cheese were significantly ( $p .001$ ) affected by the fat and protein content of the milk from which cheese was made. The moisture content of the cheese varied between vats and was affected by milk composition. For each enzyme, mean percent fat in the dry matter (FDM) in the cheese was greater in cheese made from high fat (3.91%) milk than in cheese made from low fat (3.54%) milk; protein content in the two lots of milk were similar (3.1%). The mean percent moisture in the nonfat substance (MNFS) was neither affected by milk composition nor by the milk clotting enzyme used during cheese making. Table 1 shows also the concentration of fat and protein in the whey. Milk composition and milk clotting enzyme used in cheese manufacture did not affect losses of fat and protein in the whey.

Cheese made with the different strains of S. cremoris were not significantly different in mean fat, protein, moisture, FDM, and MNFS (Table 2). Mean percent fat and protein in the whey was not affected by the strain of starter bacteria used in cheese making (Table 2).

### Cheese Yield

Wet weight and dry matter yields of cheese made with the different strains of S. cremoris are shown in Table 2. Similar

1 results for cheese made with the different milk clotting enzymes are  
2 shown in Table 3. Neither milk composition, milk clotting enzyme nor  
3 strain of starter bacteria affected the wet weight yield of cheese.  
4 Wet weight yields of cheese were between 10.42% and 10.71% for cheese  
5 made from the two lots of milk clotted with the different milk  
6 clotting enzymes. Wet weight cheese yields are 11.05% for strain ML1,  
7 10.84 for strain HP, and 10.77 for strain 30/70. The coefficient of  
8 variation of wet weight yields between replications ranged from 1.7 to  
9 3.1% for cheese made with different milk-clotting enzymes or different  
10 strains of starter bacteria. Due to variations in moisture content of  
11 cheese, it was necessary to compute dry matter yields for adequate  
12 yield comparisons. Dry matter yields were between 6.52 and 6.71% with  
13 coefficient of variations between .1 and 2.6% for cheese made with the  
14 different milk-clotting enzymes. Mean dry matter yields for cheese  
15 made with different strains of S. cremoris were 6.62, 6.64 and 6.67%  
16 for strains 30/70, HP and ML1 respectively. Neither milk clotting  
17 enzyme (Table 4) nor strain of starter bacteria (Table 5) used in  
18 cheese making affected dry matter yields significantly. However,  
19 there was a significant difference between dry matter yields of cheese  
20 made from different lots of milk (Table 4). At constant protein  
21 level, dry matter yields were higher with higher fat content of the  
22 milk.  
23  
24  
25  
26  
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28

## DISCUSSION

Dry matter yields and cheese yields adjusted to a constant moisture content, under controlled manufacturing conditions, were significantly affected by the concentration of fat and protein in the milk. The milk-clotting enzymes did not differ significantly in their effect on cheese yield. Similarly, significant differences in yield were not observed when different strains of S. cremoris were used for cheese manufacture. Careful standardization of the activities of milk clotting enzymes was necessary as an initial step to prevent yield losses. The proteolytic activities of the milk-clotting enzymes used in this study, as judged by the percent nitrogen in the whey were essentially the same.

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Table 1. Composition of constituents in whey and cheese made with different milk-clotting enzymes.

Enzyme	Milk*	Cheese					Whey	
		Protein (%)	Fat (%)	Moisture (%)	FDM (%)	MNFS (%)	Protein (%)	Fat (%)
		Mean $\pm$ standard error of mean						
Chymosin	1	23.24 $\pm$ .26	34.8 $\pm$ .5	36.33 $\pm$ .68	54.7	55.7	.913 $\pm$ .009	.4521 $\pm$ .021
	2	21.43 $\pm$ .13	31.6 $\pm$ .1	37.98 $\pm$ .65	51.0	55.5	.918 $\pm$ .006	.3711 $\pm$ .025
Bovine Pepsin	1	23.10 $\pm$ .33	34.8 $\pm$ .4	36.41 $\pm$ .54	54.7	55.8	.907 $\pm$ .004	.529 $\pm$ .034
	2	21.98 $\pm$ .28	32.1 $\pm$ .1	37.94 $\pm$ .77	51.7	55.9	.924 $\pm$ .005	.466 $\pm$ .028
Porcine Pepsin	1	22.85 $\pm$ .42	35.1 $\pm$ .3	37.39 $\pm$ .81	56.4	57.6	.904 $\pm$ .004	.450 $\pm$ .043
	2	21.42 $\pm$ .15	31.2 $\pm$ .1	38.62 $\pm$ .74	50.8	56.1	.925 $\pm$ .003	.430 $\pm$ .026
<u>Mucor miehei</u> protease	1	23.55 $\pm$ .25	35.4 $\pm$ .3	36.03 $\pm$ .85	55.3	55.8	.904 $\pm$ .006	.387 $\pm$ .014
	2	21.67 $\pm$ .17	31.5 $\pm$ .1	38.14 $\pm$ .41	50.9	55.7	.927 $\pm$ .004	.428 $\pm$ .020
<u>Mucor pusillus</u> protease	1	23.28 $\pm$ .24	35.5 $\pm$ .3	36.60 $\pm$ .36	56.0	56.7	.938 $\pm$ .010	.437 $\pm$ .031
	2	21.86 $\pm$ .30	31.8 $\pm$ .2	37.49 $\pm$ .45	50.9	55.0	.927 $\pm$ .006	.485 $\pm$ .014
<u>Endothia parasitica</u> protease	1	23.08 $\pm$ .26	34.8 $\pm$ .4	35.89 $\pm$ .58	54.3	55.0	.934 $\pm$ .001	.408 $\pm$ .031
	2	22.09 $\pm$ .21	31.3 $\pm$ .3	38.06 $\pm$ .69	50.5	55.4	.946 $\pm$ .010	.357 $\pm$ .028

\*Milk 1 contained 3.91% fat and 3.13% protein  
Milk 2 contained 3.54% fat and 3.12% protein

FDM = Fat in the dry matter  
MNFS = Moisture in nonfat substance

<sup>a</sup> Each mean represents duplicate determinations from 3 trials

Table 2. Concentration of constituents in whey and cheese and the yield of cheese made with three strains of S. cremoris.

Strain of <u>S. cremoris</u>		Whey		Cheese					Wet Weight Yield (g)	Dry Matter Yield (g)
		Protein (%)	Fat (%)	Protein (%)	Fat (%)	Moisture (%)	FDM (%)	MNFS (%)		
ML1	$\bar{X}$	.927	.395	22.11	32.2	39.60	55.0	59.3	773.4	467.1
	S.E.M.	.008	.017	.12	.4	.52	.3	.5	--	--
	CV	--	--	--	--	--	--	--	1.7	.6
HP	$\bar{X}$	.922	.469	22.58	33.7	38.70	55.0	58.4	759.0	464.8
	S.E.M.	.013	.042	.11	.5	.49	.9	.4	--	--
	CV	--	--	--	--	--	--	--	2.1	.6
30/70	$\bar{X}$	.896	.312	22.59	33.9	38.51	55.1	58.2	754.2	463.5
	S.E.M.	.004	.037	.18	.5	.77	.5	.8	--	--
	CV	--	--	--	--	--	--	--	1.9	.7

$\bar{X}$  = mean    S.E.M. = standard error of mean    CV = coefficient of variation

Each mean represents duplicate determinations on 4 trials. Mean of cheese yields are from 4 trials.



Table 3. Wet and Dry Matter Yield of Cheese from 7000.0g milk.

Enzyme	Milk <sup>a</sup>	Mean Wet Weight Yield (g)	CV <sup>b</sup>	Mean Dry Matter Yield (g)	CV <sup>b</sup>
Chymosin	1	736.1	2.7	468.4	.6
	2	749.2	1.8	464.4	1.1
Bovine Pepsin	1	738.7	1.7	469.6	2.2
	2	739.0	2.6	458.4	.7
Porcine Pepsin	1	738.2	2.2	461.8	1.0
	2	748.7	3.1	459.1	.1
<u>Mucor miehei</u> protease	1	738.4	2.7	471.8	1.8
	2	741.0	1.7	458.3	1.1
<u>Mucor pusillus</u> protease	1	732.6	2.4	468.8	2.6
	2	729.8	2.1	456.1	.4
<u>Endothia parasitica</u> protease	1	729.6	1.8	467.7	.8
	2	749.8	3.0	464.1	1.1

<sup>a</sup> Milk1 contained 3.91% fat and 3.13% protein.

Milk 2 contained 3.52% fat and 3.12% protein.

<sup>b</sup> CV = coefficient of variation.

Mean of cheese yields are from 3 trials.

Table 4. Analysis of variance tables for wet weight, adjusted and dry matter yields of cheese.

Source of variation	df	Sum of squares	Mean Square	F-ratio
<u>Wet Weight Yield</u>				
Enzyme	5	568	114	.22
Milk	1	487	487	.95
Enzyme x Milk	5	567	113	.22
Error	24	12316	513	
Total	35	13937		
<u>Adjusted Yield (37% moisture)</u>				
Enzyme	5	1107	221	.43
Milk	1	6414	6414	12.43
Enzyme x Milk	5	687	137	.27
Error	24	12382	516	
Total	35	20591		
<u>Dry Matter Yield</u>				
Enzyme	5	150.3	30.1	.5
Milk	1	571.4	571.4	9.9
Enzyme x Milk	5	190.7	38.1	.6
Error	24	1387.2	57.8	
Total	35	2299.6		

\* significant at  $<.005$ \*\* significant at  $<.005$

Table 5. Analysis of variance tables on wet weight and dry matter yields of cheese made with 3 strains of S. cremoris.

Source of variation	df	Sum of squares	Mean Square	F-ratio
Wet Weight Yield				
Culture (ML1, HP, 30/70)	2	800	400	1.39
Error	9	2592	288	
Total	11	3392		
Dry Matter Yield				
Culture (ML1, HP, 30/70)	2	25.0	12.5	1.12
Error	9	100.6	11.2	
Total	11	125.5		

Role of milk-clotting enzymes on curing of  
Cheddar Cheese - A progress report

N.A. Yiadom-Farkye and C.A. Ernstrom

A series of experiments to determine the role of milk clotting enzymes and proteases from starter bacteria on curing of Cheddar cheese was started in February 1987. To date, all the cheese has been manufactured and analyses are in progress. A brief description of the experimental design, procedures and some of the results are included.

## MATERIALS AND METHODS

### Enzymes

Five milk clotting enzymes were tested. The test enzymes were chymosin (CHY), bovine pepsin (BP) porcine pepsin, (PP), Mucor miehei protease (MM) and Mucor pusillus protease (MP).

### Cultures

The cultures tested were *S. cremoris* strain MLI, HP and a mixed 4-strain commercial culture. The abilities of the different cultures to produce bitterness in milk was tested by propagating the cultures in pasteurized whole milk at 4°C for up to 7 days. Bitterness was detected in the lot containing strain HP but not in lots containing MLI or the 4 strain commercial culture.

### Experimental Design

Experiments were designed using a combination of milk clotting enzymes, starter bacteria and setting pH as follows. Triplicate determinations were done for each series.

Series	Enzyme	pH of milk at setting	Starter
1	BP	6.6	commercial 4 strain
1	BP	6.2	commercial 4 strain
1	PP	6.6	commercial 4 strain
2	MM	6.6	commercial 4 strain
2	MP	6.6	commercial 4 strain
2	PP	6.6	commercial 4 strain
3	PP	6.6	HP
3	PP	6.6	MLI
3	PP	6.6	commercial 4 strain
4	CHY	6.6	HP
4	CHY	6.6	MLI
4	PP	6.6	commercial 4 strain
5	1PP	6.2	commercial 4 strain
5	2PP	6.2	commercial 4 strain
5	3PP	6.2	commercial 4 strain

### Grading

All the cheese have been graded at 1 month of age by a trained panel of 6 judges. Grading will be continued at 3, 5, 7 and 9 months of ripening. Judges were selected based on their abilities to detect low levels (.0008 g) of quinine sulphate in milk. Grading is for appearance, flavor, body and texture, overall, cheese flavor intensity and curdiness. A linear scale of 1 (unsaleable) to 7 (superior) is being used.

### Proteolysis

Primary proteolysis in cheese is being followed at 1, 3, 5, 7, and 9 months of ripening by disc polyacrylamide gel electrophoresis (PAGE).

Secondary proteolysis also is being followed by measuring the percent of total nitrogen soluble in water.

### RESULTS AND DISCUSSION

Because of the preliminary nature of the experiments, concrete conclusions cannot be drawn. However, there seems to be a trend in how much secondary proteolysis is occurring. (Table 1) Comparing all the enzymes to porcine pepsin which had the least proteolytic effect in cheese (based on percent water soluble nitrogen) when used in milk at pH 6.6, the proteolytic effect of the enzymes are as follows from the least to the most proteolytic.

- |                                   |        |
|-----------------------------------|--------|
| 1. Porcine pepsin                 | pH 6.6 |
| 2. Porcine pepsin                 | pH 6.2 |
| 3. 2 x porcine pepsin             | pH 6.2 |
| 4. <u>Mucor miehei</u> protease   |        |
| 5. Chymosin                       |        |
| 5. 3 x porcine pepsin             | pH 6.2 |
| 5. Bovine pepsin                  | pH 6.6 |
| 6. <u>Mucor pusillus</u> protease |        |

So far, the strain of starter organism does not seem to have an effect on secondary proteolysis. Bitterness was detected in cheese made with strain HP and also with Mucor pusillus protease.

TABLE 1. Percent water soluble nitrogen in cheese.

Enzyme	Culture	% water soluble N at	
		1 mo	3 mo
Porcine pepsin	MLI	7.88±.49	12.53±.27
	HP	7.39±.18	12.08±.33
	commercial	7.56±.51	12.89±.46
<u>Mucor miehei</u> protease	commercial	11.07±.36	19.73±1.27
<u>Mucor pusillus</u> protease	commercial	16.17±.54	27.94±1.42
Porcine pepsin	commercial	6.90±.18	11.32±.63
Chymosin	HP	11.32±.63	
Chymosin	MLI	11.28±.46	
Porcine pepsin	commercial	6.93±.18	
Bovine pepsin (6.2)	commercial	11.28±1.08	
Bovine pepsin (6.6)	commercial	11.43±1.31	
Porcine pepsin	commercial	6.99±.47	
1xPorcine pepsin	commercial	9.51±.68	
2xPorcine pepsin	commercial	10.44±.77	
3xPorcine pepsin	commercial	11.99±.42	

Enzyme	Culture	Trial	Total Flavor score* at	
			1 mo	3 mo
1 x Porcine pepsin (pH 6.2)	commercial	1	33	33
		2	28	--
		3	32	--
2 x Porcine pepsin (pH 6.2)	commercial	1	34	33
		2	29	--
		3	31	--
3 x porcine pepsin	commercial	1	31	28
		2	30	--
		3	32	--
Chymosin	MLI	1	34	33
		2	30	--
		3	29	--
Chymosin	HP	1	30	17
		2	25	--
		3	29	--
Porcine pepsin	commercial	1	29	30
		2	29	--
		3	25	--

\*The maximum total flavor score that cheese can have is 42



Enzyme	Culture	Trial	total Flavor score at		
			1 mo	3 mo	5 mo
Porcine pepsin	MLI	1	30	34	29
		2	31	33	30
		3	29	30	--
Porcine pepsin	HP	1	28	24	23
		2	33	27	22
		3	27	24	--
Porcine pepsin	commercial	1	30	28	26
		2	31	31	31
		3	30	31	--
<u>Mucor miehei</u> protease	commercial	1	31	28	--
		2	33	30	--
		3	32	31	--
<u>Mucor pusillus</u> protease	commercial	1	28	29	--
		2	28	27	--
		3	25	22	--
Porcine pepsin *	commercial	1	32	32	--
		2	28	29	--
		3	29	31	--

Enzyme	Culture	Trial	Total Flavor score at 1 mo	3 mo
Bovine pepsin (pH 6.6)	commercial	1	29	28
		2	24	19
		3	25	--
Bovine pepsin (pH 6.2)	commercial	1	32	33
		2	33	32
		3	29	--
Porcine pepsin	commercial	1	28	27
		2	30	30
		3	31	--

TITLE: Evaluation of Milk Proteins as Whitening Agents in Processed Meat and Poultry Products

Principal Investigator: Daren Cornforth, Ph.D.  
Associate Professor  
Dept. of Nutrition and Food Sciences  
Utah State University  
Logan, UT 84322-8700

PROPOSED OBJECTIVES:

- A. Determine optimum use level for various milk protein fractions in manufacture of combo light-dark veal or poultry rolls.
- B. Determine the mechanism by which caseinates, (and possibly other milk proteins) lighten the color of meat or poultry rolls containing dark meats.

SIGNIFICANCE

Sodium and calcium caseinates (1%) lighten the color of poultry rolls containing thigh meat, by an unknown mechanism. Combo poultry rolls with caseinates have a more uniform, lighter color, and sell better. Other milk protein fractions may also improve color of combo rolls, or even of rolls with all dark meat. If so, use of milk proteins in manufacture of poultry and meat rolls could increase substantially. One potentially significant use of milk proteins may be to lighten the color of veal, permitting older calves to be sold as veal.

EXPERIMENTAL APPROACH

Turkey combo rolls (70:30 light:dark meat) will be formulated to contain 0, 1, 2, 3 and 4% whey protein concentrate. Product will be evaluated by sensory panel for acceptability and color. Color will also be quantified by use of a Hunter colorimeter. PH, oxidation reduction potential and myoglobin solubility will also be measured before and after processing. Reflectance spectra of final products will also be obtained with a Hunter spectrophotometer. Pigment solubility and reflectance spectra will provide information on the type and quantity of pigment present, and thus on the effect of caseinates on product color. Similar experiments will be repeated for non fat dry milk, sodium caseinate, and calcium caseinate (control), using both turkey meat and veal.

Further model system studies of milk protein-myoglobin interactions may be of interest, depending on the results of the initial reflectance and solubility data.

# BUDGET

<u>Budget item</u>	<u>Cost</u>	<u>Cost-2nd Yr</u>
Graduate Research Assistant - 1/2 time	\$6,000	\$6,000
Boneless turkey breast meat and thigh meat		
5 rolls/treatment X 2 (duplicate) X 4 treatments X 5 lbs/roll = 470# meat X \$1.75/lb	823	--
Veal 470# X \$2.00/lb	940	
Other ingredients and processing supplies	100	100
Lab supplies computer time, etc.	\$1,964	1,412
Publication costs, travel		1,000
Lab Tech. - .25 time (including benefits)	<u>5,500</u>	<u>5,500</u>
Annual Total:	\$15,327	\$14,012

Initial work will be done in the first year. Model system studies and publications will be done in the second year.

Two Year Total: \$29,339

## OTHER COLOR PUBLICATIONS

1. Cornforth, D.P. and Egbert, W.R. 1985. Effect of rotenone and pH on the color of pre-rigor muscle. J. Food Sci. 50:34.
2. Cornforth, D.P., Egbert, W.R. and Sisson, D.V. 1985. Effects of low temperature and increased oxygen concentration on color of intact pre-rigor muscle. J. Food Sci. 50:1021.
3. Egbert, W.R. and Cornforth, D.P. 1986. Factors influencing color of dark cutting beef muscle. J. Food Sci. 51:57.
4. Orcutt, M.W., Dutson, T.R., Cornforth, D.P. and Smith, G.C. 1984. Factors affecting the formation of a dark, coarse band ("Heat-Ring") in bovine longissimus muscle. J. Ani. Sci. 58:1366.
5. Cornforth, D.P., Vahabzadeh, F., Carpenter, C.E. and Bartholomew, D.T. 1986. Role of reduced hemochromes in pink color defect of cooked turkey rolls. J. Food Sci. 51:1132.

DAIRY RESEARCH FOUNDATION

Grant Application

1987

1. Title of Proposal:

- A. For professional interpretation: Evaluation of milk proteins as whitening agents in processed meat and poultry products.
- B. For layman interpretation: Use of milk proteins to improve color of veal and turkey rolls.

2. Principal Investigator: Daren P. Cornforth

2a. Official Position: Associate Professor

2b. Mailing Address:

Office:

Home:

Department of Nutrition and Food Sciences  
Utah State University  
Logan, UT 84322-8700

56 W. 200 N.  
Providence, UT 84332

2c. Telephone No. Office: (801) 750-2114  
Home: (801) 753-0979

3. Date of Proposed Project

From January 1, 1987 through December 31, 1988

4. Total Amount Requested:

First Year: \$15,327.00

Second Year: \$14,012.00

5. Principal Investigator's Organization:

Utah State University

6. Name, Official Position, Address and Telephone Number of Administrative Official Responsible for Financial Accountability of Grant.

Doyle J. Matthews  
Dean and Director  
College of Agriculture and  
Utah Agricultural Experiment Station  
Utah State University  
Logan, Utah 84322-4800  
(801) 750-2213

PROJECT TITLE: Use of milk proteins to improve color of veal and turkey rolls.

7. Name, Official Position, Address and Telephone Number of Administrative Official Responsible for Financial Accountability of Grant:

M. Kay Jeppeson  
Director, Contracts & Grants  
Contract and Grant Office  
Utah State University  
Logan, Utah 84322-1400  
(801) 750-1226

8. Name, Official Position and Department of All Professional Personnel Involved in Proposed Project:

Daren P. Cornforth  
Associate Professor  
Department of Nutrition and Food Sciences  
Utah State University  
Logan, UT 84322-8700  
(801) 750-2114

PROJECT TITLE: Use of milk proteins to improve color of veal and turkey rolls.

#### 10. Project Details

##### a. Objectives:

The objective of the proposed research is to determine optimum use level of various milk protein fractions in manufacture of combo light-dark meat and poultry rolls. Caseinates lighten the color of poultry rolls containing dark meat. Light colored rolls sell better.

Thus, a second objective of the proposed work is to determine the mechanism by which milk proteins lighten the color of poultry rolls. If other milk protein fractions also lighten poultry rolls, the use of milk proteins in manufacture of meat and poultry rolls could increase substantially.

##### b. Background information:

Milk proteins are currently permitted in nonspecific red meat products for emulsion stability, water and fat binding, and to improve texture. In poultry products containing dark (thigh) meat, an additional and very important attribute of caseinates is a lightening effect. This effect has only recently been reported (Andres, 1986), and the mechanism for the whitening effect has not been investigated. Thus, it is very important to learn more about the cause of the whitening effect, and especially to determine if milk protein fractions other than caseinates also cause whitening of poultry loaves.

The level of caseinates, whey protein concentrate, or nonfat dry milk is limited to 2%, 3.5%, or 2%, respectively, in products such as

PROJECT TITLE: Use of milk proteins to improve color of veal and turkey rolls.

hot dogs, but is permitted at levels sufficient to obtain desired functionality in non-specific meat loaves (deHoll, 1981). If whey proteins or nonfat dry milk also cause whitening of veal or poultry loaves, it will be necessary to determine optimum use levels. For all milk protein fractions (including caseinates) that cause whitening, it will be of great interest to determine the ratio of milk protein: dark meat sufficient to cause whitening. For example, 1% calcium caseinate may be sufficient to cause whitening in combo rolls containing 10% dark meat, but 3% caseinate may or may not be sufficient to cause whitening in combo rolls containing 30% dark meat. It will also be of interest to determine the possibility of using milk proteins to produce an acceptable light-colored poultry roll from 75 or 100% dark meat.

c. Design of Proposed Project - include Grant Chart (use attached or design your own) analytical and statistical methods for evaluation:

Comparison of whitening effect of various milk proteins in veal or poultry combo rolls. Veal or turkey combo rolls (75:25 light:dark meat) will be formulated to contain 0, 1, 2, 3, or 4% sodium caseinate, calcium caseinate, nonfat dry milk (NFDM), or whey protein concentrate (WPC). If whitening is observed in 75:25 rolls, further product will be prepared at 50:50, 25:75 and 0:100 light:dark meat. If little or no whitening is observed at the 75:25 ratio, further product will be prepared at 90:10 light to dark meat.



PROJECT TITLE: Use of milk proteins to improve color of veal and turkey rolls.

The initial trial at 75:25 light to dark meat will consist of 4 milk protein treatments (sodium and calcium caseinate), NFDM, and WPC, 5 concentrations (0,1,2,3,4%) in duplicate for a total of 40 turkey rolls. At 5 lbs./roll, 200 lbs. turkey meat is required (150 lbs. light and 50 lbs. dark meat).

For comparison, 2 rolls consisting of 100% light meat will also be prepared. For further experimentation at 10, 50, 75, or 100% dark meat, another 200-250 lbs. of turkey meat may be required, depending on the effectiveness of the various milk proteins for causing whitening in the initial trial.

Thus, about 470 lbs. turkey meat will be required. Turkey meat will be obtained directly from a local processor, and turkey rolls will be prepared by standard industry procedures (Cornforth et al, 1986).

Product will be evaluated by a 10-member, trained sensory panel for acceptability and color. Palatability parameters of tenderness, juiciness, and flavor will be determined with a 9 point scale, where 9= very tender, juicy, or desirable, and 1= very tough, dry, or undesirable, respectively. Appearance characteristics of color and uniformity of color will also be evaluated on 1 cm thick slices, using a 9 point scale, where 9= very light or very uniform and 1= very dark, or very spotty, respectively. Color will also be measured with a Hunter Color Meter (Cornforth and Egbert, 1985).

The whitening effect of caseinates on meat color could be due to

PROJECT TITLE: Use of milk proteins to improve color of veal and turkey rolls.

a masking effect of the opaque caseinates, or to a direct interaction with or oxidation of the heme pigment itself. To quantify such effects, pH, oxidation-reduction potential, and reflectance spectra of cooked products will be obtained (Cornforth et al, 1986). Myoglobin solubility of cooked products will also be determined (Krzywicki, 1982). Pigment solubility and reflectance spectra will provide information on the type and quantity of pigment present, and thus provide information on the effect of milk proteins on meat color.

Further model system studies on milk protein-myoglobin interactions may be warranted, if initial results indicate that such interactions indeed occur.

PROJECT TITLE: Use of milk proteins to improve color of veal and turkey rolls.

#### Project Timetable

The graduate research assistant will have primary responsibility for phase I, the preparation of the 75:25 light:dark meat rolls. This and the associated chemical analysis will take 8 months. Phase II, the preparation and analysis of turkey rolls with varying light:dark meat ratios will take an additional 8 months. A student technician will assist with phases I and II.

The principal investigator (D.P.C.) will assist with phases I and II, and will have primary responsibility for phase III, analysis and preparation of results for publication, during the final 8 months of the 2-year funding period.

#### References

1. Andres, C. 1986. Milk protein technology for red meat also applicable to poultry and fish. *Fod Processing* 47(5):58.
2. Cornforth, D.P. and Egbert, W.R. 1985. Effect of rotenone and pH on the color of pre-rigor muscle. *J. Food Sci.* 50:34.
3. Cornforth, D.P., Vahabzadeh, F., Carpenter, C.E. and Bartholomew, D.T. 1986. Role of reduced hemochromes in pink color defect of cooked turkey rolls. *J. Food Sci.* 51:1132.
4. deHoll, J.C. 1981. *Encyclopedia of Labeling Meat and Poultry Products*, 5th Ed. Meat Plant Magazine, St. Louis, Mo.
5. Krzywicki, K. 1982. The determination of haem pigments in meat. *Meat Sci.* 7:29.

PROJECT TITLE: Use of milk proteins to improve color of veal and turkey rolls.

d. Available Facilities and Equipment:

Product preparation: The USU meats lab, housed in the new (1974) Nutrition and Food Sciences building, is spacious and well-equipped with all equipment needed for preparation of meat rolls, including a 20-liter bowl chopper, mixers, grinder, hydraulic sausage stuffer, and smokehouse with wet and dry bulb temperature controls.

Product analysis: The NFS meat science labs are well-equipped with all instrumentation necessary for muscle pigment analysis, including Orion pH meter with ORP electrode, Hunter Lab Digital Color Difference Meter Model D25D2A, Beckman spectrophotometer, analytical and top loading balances.

e. Significance of the Project to the Dairy Industry

Per capita consumption of poultry meats is rapidly increasing in the U.S. However, rolls and loaves containing dark (thigh) meat do not sell as well as the lighter and more uniform breast meat products. Combo light:dark meat rolls with caseinates also have a uniform, light color, and sell better than rolls formulated without caseinates. Other milk protein fractions may also improve color of combo rolls, or even rolls made with all dark meat. If so, use of milk protein in manufacture of poultry and meat rolls could increase substantially. One potentially significant use of milk proteins may be to lighten color of veal products, permitting older calves to be sold as veal.

PROJECT TITLE: Use of milk proteins to improve color of veal and turkey rolls.

Professional Experience (related to proposed project):

1971-1974 - M.S. research at Colorado State University on effects of breed, sex, and diet on bovine muscle growth and muscle fiber type.

1974-1978 - Ph.D. research on relationship of mitochondrial and sarcoplasmic reticulum membranes to cold shortening in beef and rabbit muscle.

1978-present - Assistant and Associate Professor at Utah State University. Research has focused on meat color chemistry and associated meat color defects in turkey and beef muscle, and on mechanism of nitrite inhibition of botulism in temperature-abused, cured meats.

Publications Related to Proposed Project:

1. Cornforth, D.P., W.C. Schwartz and D.A. Cramer. 1973. Growth and differentiation of muscle fibers in cattle. Proc. Western Section, Amer. Soc. Animal Sci. 24:106.
2. Spindler, A., D.P. Cornforth, D.A. Cramer, M.M. Mathias and A. L. Hecker. 1973. The effects of growth, breed, and sex on the growth and differentiation of muscle fibers in cattle. 86th Annual Research Conference, Colo. State University 86:20.
3. Cramer, D.A., A.L. Hecker and D.P. Cornforth. 1973. The effects of growth, breed and sex on the deposition and distribution of fat in cattle. 86th Annual Research Conference, Colo. State University 86:70.
4. Kanda, T., A.M. Pearson, M.A. Porzio and D.P. Cornforth. 1977. Comparison of isolated sarcoplasmic reticulum from bovine and rabbit muscle. Meat Sci. 1:253.
5. Porzio, M.A., A.M. Pearson and D.P. Cornforth. 1979. M-Line protein: Presence of two non-equivalent high molecular weight components. Meat Sci. 3:31.
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PROJECT TITLE: Use of milk proteins to improve color of veal and turkey rolls.

Certification and Acceptance: We, the undersigned, certify that the statements contained herein are true and complete to the best of our knowledge and accept, as to any grant awarded, the obligation to comply with the terms in Dairy Research Foundation Memorandum of Agreement in effect at the time of the award.

14. Signature of Principal Investigator:

Date \_\_\_\_\_

15. Signature of Official of Principal Investigator's Organization:

Date \_\_\_\_\_



**Project Title:** Evaluation of Milk Proteins as Whitening Agents in Processed Meats and Poultry Products.

**Personnel:** D. Cornforth, Associate Professor, Nutrition and Food Sciences, Utah State University

Brent Dobson, Graduate Student, Nutrition and Food Sciences, Utah State University

**Funding Sources:** Western Dairy Foods Research Center

### Results:

Bologna (90% turkey thigh meat, 10% fat), turkey rolls (100% turkey thigh meat) and patties (100% turkey breast meat) were formulated with 3% nonfat dry milk (NFDM), 3% calcium caseinate (as percent of final product), or controls. Bologna made with 3% NFDM was significantly lighter and less red than controls, by both visual appraisal and reflectance measurement with a Hunter colorimeter. White meat patties made with 3% NFDM or 3% caseinate were also significantly lighter than controls. No whitening effect was observed for thigh meat rolls containing milk proteins.

Dramatic whitening of turkey thigh meat or mechanically deboned turkey was achieved in production of turkey surimi, in which meat was ground, and muscle pigments extracted in 0.04 molar phosphate buffer, pH 8.0. An acceptable turkey nugget was produced using 50% white meat and 50% washed, mechanically deboned turkey meat.

Milk proteins appear to have greatest whitening effect in emulsified products (bologna) or meat with low pigment content (turkey breast patties).

### Impact of Research:

Sodium and calcium caseinates (1%) have previously been reported to lighten the color of thigh meat poultry rolls. Combination light-dark meat poultry rolls formulated with caseinates have a lighter, more uniform color, and sell better. Present results indicate that NFDM also lightens color of turkey thigh meat bologna. NFDM may also lighten color of turkey rolls, if an injection-tumbling procedure is used to better distribute the NFDM. If so, use of milk proteins in poultry processing could increase substantially. Another potentially significant use of milk proteins may be to lighten the color of veal.

The work on the incorporation of milk proteins, as whitening agents, in meat emulsions is progressing well. To date we have seen lightening in ground turkey breast and a bologna type product, when milk proteins are added.

The further processing of turkey (ie. pre-cooked products) has increased significantly in the U.S. in the last few years. Today the industry processes 1.75 million tons per year (National Turkey Federation, 1989). If milk proteins were used in all processed products at a three percent level, utilization of milk proteins would be 52,500 tons/year. The dollar value at possible usage levels

could be \$94,500,000.00 per year using nonfat dry milk with a price of \$0.90 per pound. However if the meat industry used caseinates which run \$2.85 - \$3.10/pound this dollar amount could increase to \$299,250,000 - \$325,500,000 respectively.

**Project Title:** Effects of calcium caseinate, non-fat dry milk, and whey protein concentrate on color and texture of turkey rolls

**Personnel:** Daren P. Cornforth, Dept. of Nutrition and Food Sciences, Utah State University

Brent N. Dobson, Graduate Students, Dept. of Nutrition and Food Sciences, Utah State University

**Funding Sources:** Western Dairy Foods Research Center  
USU Agricultural Experiment Station  
Rite-wood Farms, Franklin, Idaho

### **Objectives:**

To determine the effects of various milk proteins (3% calcium caseinate, non-fat dry milk (NFDM), or whey protein concentrate (WPC)) on color, texture and panel acceptability of turkey rolls containing 10 or 30% turkey thigh meat.

### **Results:**

The turkey rolls were evaluated by a trained sensory panel (21 panelists) and by instrumentation. Color intensity, color uniformity, cohesiveness, tenderness, flavor, juiciness and overall acceptability were evaluated on a 7 point scale, where 7 was high and 1 was low for each attribute. The study was repeated three times. Controls (no milk proteins) and rolls formulated with NFDM or WPC were rated significantly higher than rolls containing caseinate for color uniformity, cohesiveness, flavor and overall acceptability. Rolls containing caseinate were significantly darker than controls or rolls with WPC. Panelists detected no significant differences among milk protein treatments for juiciness or toughness. However, the rolls formulated with 10% thigh meat were significantly more tender than rolls formulated with 30% thigh meat. Instrumental analysis (Hunter color and succulometer ) also showed no significant differences among milk protein treatments for color or expressible juices, respectively. Rolls made with milk proteins had significantly higher cooked yields (89%) than control rolls (86%).

In conclusion, milk powders containing lactose (NFDM and WPC) increased the yield and cohesiveness of turkey rolls. color was unaffected, compared to controls. Turkey rolls made with calcium caseinate also increased yield compared to controls, but the rolls had poor texture and insufficient cohesiveness.

### **Impact of Research:**

Turkey rolls and other precooked meat items such as ham, bacon, or roast beef usually contains 0.5% phosphate to increase the cooked yield and product cohesiveness. Results of this study showed that addition of 3% NFDM or WPC increased yield and improved texture of turkey rolls, even in the absence of phosphate. Calcium caseinate addition increased yield, but texture was poor. NFDM or WPC are both over 50% lactose. Calcium caseinate contains no lactose. It thus appears that lactose is the substance that improves texture of turkey rolls.

Phosphates are not permitted in some cooked meat products in Europe or Japan. Thus there is great interest in substitutes for phosphates in precooked meats. Based on results of this study, both NFDM and WPC have potential to replace phosphate in precooked meats.

Caseinates have been the most widely promoted milk powder for use in meat products. Results of this study show that lactose-containing NFDM and WPC were actually superior to calcium caseinate in a cooked turkey roll. Thus there is great potential for increased use of NFDM and WPC or lactose in precooked poultry products.

### **Publications and Abstracts:**

Dobson, B.N., and D.P. Cornforth. 1990. Effects of calcium caseinate, nonfat dry milk, and whey protein concentrate on color and texture of turkey rolls. Proceedings of 43rd Annual Reciprocal Meat Conference, Starkville, Mississippi (Abstract).

Dobson, B.N., Cornforth, D.P., and Pyke, D. 1990. Effects of calcium caseinate, nonfat dry milk, and whey protein concentrate on color and texture of turkey rolls. Journal of Food Science (in preparation).

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MAY 16 1987

Dairy Board Proposal

**Iron Fortification of Cheese Curd**

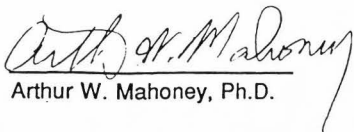
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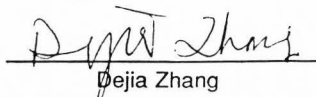
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Dejia Zhang

May 15, 1987

## INTRODUCTION

Most dairy products are excellent sources of protein, vitamins especially riboflavin, vitamin D when added, minerals especially calcium, and energy. The one major nutrient that is deficient in dairy products is iron. Iron deficiency continues to be a major health problem in the U.S. as well as the world. For example, Dr. Hendricks recently found that 22 of 50 USU college women tested had serum ferritin concentrations  $\leq 15$  ng/ml. (Any subject with a ferritin value below 20 ng/ml is clinically iron deficient.) Dairy products are being promoted as good sources of calcium (and vitamin D) for the prevention of osteoporosis. If suitable sources of iron were found that could be used to fortify them, dairy products could also be promoted as good sources of iron.

Osteoporosis and iron deficiency are both common health problems of women. Children under two years of age are also susceptible to iron deficiency as well as men above age 65. Many older caucasians become somewhat intolerant of lactose in fluid milk but are able to consume cultured dairy products without difficulty. **Iron fortified dairy products could be promoted for preventing iron deficiency anemias** that are prevalent in very young children, women of child-bearing age and elderly men **and osteoporosis** in both women and men.

There are a number possible strategies for fortifying dairy products. However, the addition of iron to cultured products is very intriguing because of their lactic acid content. Calcium lactate is a highly bioavailable form of supplemental calcium. The lactate salt of iron may be an excellent form of supplemental iron which could be formed naturally or on iron addition to the fresh curd subsequent to culturing. Iron fortification of milk has been studied but there are many more forms of supplemental iron that may be considered (Table 1-3). There are many potential opportunities for fortifying dairy products with iron: (1) fluid milk could be fortified by adding iron before or after homogenation and/or pasteurization, (2) yogurt and cultured butter milk could be fortified by adding iron before or after culturing, (3) natural cheese could be fortified by adding iron to the curd, perhaps with the salt, after culturing, or (4) various processed cheeses could be fortified by adding the iron before,

during or after cooking. Certainly, **iron fortification of cultured dairy products** that include yogurt, butter milk and natural and processed cheeses **is a fruitful area of product development research.**

The objective of the proposed research is to study iron fortification in cheese curd. The first part of the study is **to identify potential iron fortification sources** that will not adversely affect product quality. The initial studies will be using iron sources that are readily available. Some of these sources have been identified in Table 1; however, additional ones will be found and considered. The second part of the study is **to evaluate the bioavailability of the potential iron sources** in cheese curd that have been found to be compatible with cheese production. Although the methods described below are for milk and cheese curd, an alternate approach may be to study iron fortification of process cheese.

Table 1. Summary of iron sources that have been studied or may potentially be used in dairy products

Iron salts	Bioavailability *	Chemical Change in Dairy Products			References
		Flavor	TBA	Color	
<u>I. Simple Iron Salts</u>					
FeSO <sub>4</sub>		change,WM <sup>a</sup>	high		Wang & King, 1973a
		change after 1d,MVM			Scanlan & Shipe, 1962
			high		Hegenauer et al., 1979
		change 1d, better 14d,CM		change	Douglas et al., 1981
		change after 1 day, WM,SM			Demott, 1971
		change 1d(10mg/qt),7,14d better,WM			
		strong oxidized flavor at 40mg/qt			
					Edmondson et al., 1971
		59-iron abs. 40-79% in milk, rat			Gruden, 1982
		59-iron abs. (25ug/ml) 24%, rat			Momcilovic & Kello, 1979
	abs. 2.9-5.1% MBIF, human				
	abs. 5.9-11.3% (+Vit C) MBIF, human			Stekel et al., 1986	
	59-iron abs. 12%,WM, mice			Lonnerdal et al., 1985	
	HRE 54%, MCM, rat			Anderson et al., 1972	
	HRE 67-82%, WGM, rat			Park et al., 1986	
	59-iron abs. 22%,NFM,mice;59%,chick			Carmichael et al., 1975	
FeCl <sub>3</sub>		change (20ppm), SM			
		no change in SMC			
		no change in NDM (6mo, 27C)			
	RBV 106% in NDM, rat				Kurtz et al., 1973
FeCl <sub>2</sub>		change (20ppm), SM			
		slight change, NDM			
			high		Kurtz et al., 1973
	59-iron abs. 12%(RBV 100%),WM, mice				Hegenauer et al., 1979
					Lonnerdal et al., 1985



<u>Iron salts</u>	<u>Bioavailability</u>	<u>Chemical Change in Dairy Products</u>			<u>References</u>
		<u>Flavor</u>	<u>TBA</u>	<u>Color</u>	
Ferric sulfate no report		slight change after 5d,MVM			Scanlan and Shipe, 1962
Ferric phosphate RBV 31%(6mo store), 19%(12mo store),MBP,rat 58-iron abs. 24% (RBV 104%),cocoa drink,human abs. 35%, semi. diet, rat		minor off flavor, WM,SM			DeMott, 1971 Clemens, 1981 Fairweather-Tait et al.,1983 Morris & Ellis, 1976
<u>II. Metallic Iron</u>					
Electrolytic iron (EL) RBV 90%(6 mo store),107%(12 mo store),MBP,rat					
		no report			Clemens, 1981
Carbonyl iron (CI) RBV 119%(6mo store),69%(12mo store),MBP,rat HRE 28% (RBV 52%), MCM, rat					
		no report			Clemens, 1981 Anderson et al., 1972
Bio-Plex TM Fe		high oxidized flavor,WM	high,7.80(ctr1.74)		Baldwin et al., 1982
<u>III. Complex Iron Salts</u>					
Ferric ammonium sulfate (FeAS) abs. 10% in MP, human		change,WM slight change after 5d,WM	high		Wang & King, 1973a Scanlan & shipe, 1962 Davis et al., 1976

Iron salts	Bioavailability	Chemical Change in Dairy Products		References
		Flavor	TBA	Color
Ferrous ammonium sulfate		change after 1d,MVM no change (10mg/qt), WM distinct oxidized flavor at 40mg/qt		Scanlan & Shipe, 1962  Edmondson et al., 1971 Morris & Ellis, 1976
	abs. 35%, semi. diet, rat			
Ferric ammonium citrate (FeAC)		no change,WM no change no effect on dispersity no change (10mg/qt), WM strong rancidity flavor (40mg/qt) off flavor (10mg/qt),WM,7d rancidity change in 1d, WM,SM no change in NDM (6 mo, 27C)		Wang & King, 1973  Schoppet et al., 1974  Edmondson et al., 1971  Jones et al., 1975 DeMott, 1971
	RBV 100% (20ppm),NDM,rat RBV 107% radioiron balance 35%, pig	no change, CM		Kurtz et al., 1973 Douglas et al., 1981 Wang & King, 1973b
Ferric choline citrate (FeCC)		slight change,WM no change,CM off flavor (10mg/qt) 1d,WM after 7, 14d, better	medium high	Wang & King, 1973a Douglas et al., 1981  Edmondson et al., 1971
	RBV 102%		change,CM	

Iron salts	Bioavailability	Chemical Change in Dairy Products			References
		Flavor	TBA	Color	
Ferric citrate		no change, CM slight rancid and oxidized flavor at 40mg/qt,WM	medium high	change,CM	Hegenauer et al., 1979a Douglas et al., 1981
	RBV 122% in MBIF, rat 59-iron abs. 9%(RBV 75%),WM,mice				Edmondson et al., 1971 Theuer et al., 1973 Lonnerdal et al., 1985
Ferrous citrate					
	RBV 148% in MBIF, rat	no report			Theuer et al., 1973
Citrate phosphate iron complex (Fe, 16.7%)					
	RBV 99%, rat	no report			Ranhotra et al., 1981
Ferric pyrophosphate soluble		no change in commercial equipment treat after 5d, MVM Vit C decreased to half(13.1 to 5.7mg/l) (control 13.1 to 11.0mg/l)			
Ferric pyrophosphate		minor off flavor, WM,SM			Scanlan & Shipe, 1962 DeMott, 1971 Theuer et al., 1973
	RBV 71-125% in MBIF, rat				
Ferric glycerophosphate		no change, CM		no change,CM	
	RBV 93%	no change (10mg/qt), WM distinct rancid flavor at 40mg/qt			Douglas et al., 1981
	RBV 135% in MBIF, rat RBV 100%, SM, preschool children				Edmondson et al., 1971 Theuer et al., 1973 Layrisse et al., 1973

Iron salts	Bioavailability	Chemical Change in Dairy Products			References
		Flavor	TBA	Color	
Ferripolyphosphate (FIP)		no change,CM		no change,CM	
RBV 60%					Douglas et al., 1981
FIP (aqueous)		off flavor(10mg/qt),7d, WM			Jones et al., 1975
Sodium ferric pyrophosphate (SFP)		no change, CM		no change,CM	
RBV 35%,CM, rat; RBV 19%					Douglas, et al., 1981*
RBV 39-60% in MBIF, rat					Theuer et al., 1973
HRE 28% (RBV 52%),MCM, rat					Anderson et al., 1972
Ferric NTA (Ferric nitrilotriacetate)			low		Hegenauer, et al., 1979a
59-iron abs. 15%(RBV 125%),WM, mice					Lonnerdal et al., 1985
59-iron abs. 24%(RBV 111%),NFM,mice;69%(RBV 117%),chick					Carmichael et al., 1975
Ferric EDTA			low		Hegenauer et al., 1979a
59-iron abs. 10%(RBV 83%),WM, mice					Lonnerdal et al., 1985
59-iron incorporate to Hb,water,12%,human;4.6%,swine					Candela et al., 1984
Ferrous gluconate		change (20mg/qt), SM			
		no change, NDM			
RBV 97%		change, 1d; better 14d, CM		change,CM	Kurtz et al., 1973
		change after 1 day, WM,SM			Douglas et al., 1981
		slight change (10mg/qt), 1d			DeMott 1971
		better after 7 & 14d			
		strong oxidized flavor at 40mg/qt			Edmondson et al., 1971

Iron salts	Bioavailability	Chemical Change in Dairy Products			References
		Flavor	TBA	Color	
Ferric gluconate RBV 139% in MBIF, rat		no report			Theuer et al., 1973
Ferric lactobionate 59-iron abs. 17% (RBV 142%),WM, mice			low		Hegenauer et al., 1979a Lonnerdal et al., 1985
Ferric fructose RBV 80% 59-iron abs. 22.5%(RBV 103%),NFM,mice;61%(RBV 103%),chick			high		Hegenauer et al., 1979a Fritz et al., 1975 Carmecheal et al., 1975
Ferrous fumarate RBV 95%		slight change after 14d,CM off flavor at 10mg/qt,WM		change,CM	Douglas et al., 1981 Edmondson et al., 1971
Ferrous lactate RBV 118% in MBIF, rat		change,1d; better after 14d,CM change after 1d, WM,SM		change,CM	Douglas et al., 1981 DeMott 1971 Theuer et al., 1973
Ferric lactose		no change(125ppm in milk)		slight change with Vit C, no change	Kiran et al., 1977

Iron salts	Bioavailability	Chemical Change in Dairy Products		References
		Flavor	TBA	Color
Ferric saccharate		no fat oxidation, IC		
RBV 79-102%, rat		no off flavor, IC (highest level used, 18.5mg/100g)		darken color
				Hurrell, 1985
Ferrous succinate				
RBV 120%, rat & human		no report		Hurrell, 1985
Ferric phytate				
abs. 35% (RBV 97-102% to FeAS), semi. diet, rat		no report		Morris & Ellis, 1976
Ferric polymaltose complex				
59-iron abs. 18-29% (RBV 63%), solution, human		no report		
59-iron abs. 19-40% (RBV 78%), solution, rat				Jacobs, 1987
Ferastral (Iron-poly(sorbitol-gluconic acid) complex)				
Hb incorporation 63-111%, intramuscular injection, human		no report		Norrby & Solvell, 1977

#### IV. Complex Iron Bound to Protein

Ferripolyphosphate-whey protein complex (FIP-PRO)				
RBV 95%, rat; RBV 92%	no change, CM		no change, CM	Douglas et al., 1981
RBV 92-100%, WM, rat & chick	no change, WM			Jones et al., 1975

Iron salts	Bioavailability	Chemical Change in Dairy Products			References
		Flavor	TBA	Color	
Ferritin	abs. 0.7% in water, women abs. 0.4% in maize porridge, women abs. 12.1%,maize porridge+100mg Vit C,women		no report		Derman et al., 1982
Iron-casein	59-iron abs. 9-13% (RBV 95%), NFM, mice		no report		Carmichael et al., 1975

<sup>a</sup>ABBREVIATIONS

SM= skim milk

MP= milk powder

MCM= milk cereal mix

d= day

abs.= absorption

semi.= semipurified

WM= whole milk

MBIF= milk based infant formula

SMC= skim milk concentrate

HRE= hemoglobin regeneration efficiency

RBV= relative bioavailability value

TBA= thiobarbituric acid reaction

CM= chocolate milk

IC= infant cereal

ctr= control

NDM= non fat dried milk

MVM= multivitamin mineral milk

MBP= milk based product

NFM= non fat milk

WGM= whole goat milk

## Lactoferrin

Lactoferrin is a single chain, iron binding glycoprotein, partly saturated with  $\text{Fe}^{3+}$ . It is capable of binding two iron atoms/protein molecule and it may contain different sugars (Blanc, 1981).

Table 2. Iron, lactoferrin contents and bioavailability in human, bovine and goat milk

Item	Human milk	Cow's milk	Goat milk	Reference
Iron, mg/l	0.8	0.5	1.0	(1) (2)
Lactoferrin, g/l	1.7	traces		(3)
Major iron-binding protein	Xanthine oxidase	Casein		(4)
Bioavailability				
59-iron incorporated to Hb, adult	20.8%	13.6%		(5)
Calculated abs., new born	70%	30%		(6)
HRE(RBV), WM, rat		13.1%(14%)	50.6%(54%)	(7)
HRE(RBV), SM, rat		13.0%(14%)	26% (28%)	(7)
Absorption, MBIF, women		5.3-19.5%		(8)

(1)Blanc,1981 (2)Fomon et al., 1974 (3) Lawrence,1985 (4) Lonnerdal et al., 1981  
 (5) McMilla et al.,1976 (6) Saarinen et al.,1979 (7) Park et al., 1986 (8) Gillooly et al., 1984



Table 3. Amino acid and carbohydrate contents of lactoferrin in human milk and bovine milk\*

	Human milk	Bovine milk
Asp & Asn	65	64
Thr	33	34
Ser	46	40
Glu & Gln	69	66
Pro	33	32
Gly	49	48
Ala	56	64
1/2 Cys	26	18
Val	43	44
Met	5	5
Ile	16	16
Leu	54	62
Tyr	20	20
Phe	28	26
Lys	39	49
His	10	10
Arg	38	36
Trp	11	15
Galactose	4	5-6
Mannose	6	15-16
Fucose	2	-
GlcNAc	8	10-11
NeuAc	3	1
N-terminal	Gly	Ala
C-terminal	...	...
Molecular weight	76,400	86,100

\*Unit: mol/mol protein. Cited from Blanc, 1981.

## MATERIALS AND METHODS

### Experiment I. Screen Iron Sources for Milk and Cheese Fortification

#### 1. Iron Sources

This experiment is designed to find the iron sources which have minimal oxidative reaction with milk and cheese. The iron salts to be evaluated in this study are: 1. iron salts which cause minimal oxidative damage when added to milk or milk products; 2. iron salts which are likely not to cause oxidative damage due to their chemical structure.

#### 2. Experimental Design

Various selected iron salts will be added to milk and cheese. The milk and cheese will be stored for different time periods and then the oxidative damage of added iron salts to milk and cheese will be evaluated by taste panel judges and TBA reaction measurement. The judging panel is designed as follows:

Table 4. Experimental Design of judging panel for milk and cheese curd flavor and appearance changes caused by iron fortification

Days stored	milk			cheese		
	1d	7d	14d	1d	15d	30d
No iron added	50	50	50	50	50	50
FeSO <sub>4</sub>	50	50	50	50	50	50
Test iron I	50	50	50	50	50	50
Test iron II	50	50	50	50	50	50
Test iron III	50	50	50	50	50	50
Test iron IV	50	50	50	50	50	50
Test iron V	50	50	50	50	50	50
Test iron VI	50	50	50	50	50	50
Test iron VII	50	50	50	50	50	50
Test iron VIII	50	50	50	50	50	50

About eight iron salts are planned to be judged. With control and reference products a person will judge 10 samples at one setting in the taste panel. Approximately, **fifty people** are expected to attend each panel. Product appearance and flavor will be judged. **Charlotte Brennard** has agreed to

consult with us and assist in conducting the taste panel evaluations.

The experimental design for TBA measurement for evaluation of oxidative damage is shown in table 5. This design is similar to the statistical design used for taste panel evaluation (table 4).

Table 5. Experimental design for TBA measurement for evaluating the effect of addition of various iron salts on oxidative damage to milk and cheese curd

Days stored	milk			cheese		
	1d	7d	14d	1d	15d	30d
No iron added	3	3	3	3	3	3
FeSO <sub>4</sub>	3	3	3	3	3	3
Test iron I	3	3	3	3	3	3
Test iron II	3	3	3	3	3	3
Test iron III	3	3	3	3	3	3
Test iron IV	3	3	3	3	3	3
Test iron V	3	3	3	3	3	3
Test iron VI	3	3	3	3	3	3
Test iron VII	3	3	3	3	3	3
Test iron VIII	3	3	3	3	3	3

Triplicate samples in each treatment milk or cheese are going to be analyzed for malonaldehyde value.

### 3. Procedure

Iron salts will be put in milk after pasteurization. Most of the milk will be used to make cheese and a small part of the milk will be stored in a refrigerator for 1, 7 or 14 days. After each storage period the milk samples will be tested by TBA reaction for oxidative damage and judged by panel for flavor and color change. The milk going to cheese will be incubated with cheese starter, allowed to clot, cut, cooked and then drained of whey. The cheese curd will be dried and packaged (Kosikowski, 1966). All cheese making will be under the instruction of **Drs. Ernstrom, Richardson or Brown**. The cheese curd will be stored in a refrigerator for 1, 15 or 30 days. After each storage period, the cheese samples will be

tested by the TBA method and judged by a panel for flavor and color changes.

#### 4. Chemical and panel analysis

Fresh cheese curd will be measured for moisture, fat, protein and iron contents. Moisture will be measured by the AOAC method (AOAC, 1984) or microwave method (Richardson & Ernstrom, 1979). Fat contents will be tested by Mojonnier method (Richardson & Ernstrom, 1979). Protein contents will be determined by microKjeldahl method in a Tecator Kjelttec Auto 1030 Analyzer after digestion of cheese samples. Iron contents will be determined by colorimetry(AOAC, 1984).

A distillation method for the quantitative determination of malonaldehyde will be used to evaluate the oxidation damage of added iron to milk and cheese curd. Malonaldehyde will be condensed with TBA and then tested in a spectrophotometer at a 538 m $\mu$  wavelength (Tarladgis et al., 1960). The taste panel will also be asked to judge the flavor of milk and cheese using the scores given by Nelson and Trout (1951). Any color change after addition of iron salts to milk or in the cheese made from milk, will be judged by the panel using preference scores compared with milk or cheese with no iron added.

### Experiment II. Determination of the Bioavailability of A Iron Supplemented Milk and Chesse Curd

#### 1. Experimental Design

The iron salts selected in experiment I will be evaluated for their bioavailability in this experiment. The design of the study is as follows:

Table 6. Bioavailability of various iron salts used to fortify milk and cheese curd measured in anemic rats

Iron Sources	milk	cheese
No iron added	10	10
FeSO <sub>4</sub>	10	10
Selected iron I	10	10
Selected iron II	10	10

Selected iron III	10	10
Selected iron IV	10	10
Selected iron V	10	10

---

Ten rats are used in each group.

## 2. Diet Formulation

Milk or cheeses fortified with different iron salts will be freeze dried and mixed with other food ingredients to make different diets which will be adequate for all nutrients but iron. To determine its maximal utilization, iron must be the only dietary factor limiting hemoglobin regeneration. The amount of protein, fat and fiber will be formulated to be similar in all diets by adjusting the levels of casein, corn oil and cellulose. The final iron concentration of each diet will be approximately 30ppm and will be confirmed by chemical analysis (AOAC, 1984).

## 3. Animal experiment

Male, weanling, Spregue-Dawley rats will be individually housed in stainless steel cages with wire-mesh bottoms and fronts. Housing is in a temperature controlled room with a 12-hour day and night light cycle in the USU Laboratory Animal Research Center which is an American Association for Accreditation of Laboratory Animal Care (AAALAC) accredited facility. Animals will be made anemic by feeding the milk or cheese diet with no added iron and by bleeding approximately 1 ml of blood from the retro-ocular capillary bed twice, 3 days apart, during the 7-day pretreat period. Then the rats will be assigned randomly to treatment groups of 10 animals each balancing across treatments for hemoglobin concentration and body weight. Nine grams of test diet will be weighed and fed daily to each rat for 10 days. Spilled unconsumed foods (orts) will be weighed and recorded to determine the net consumption of the diet. Demineralized water will be offered ad libitum. Blood samples will be taken from the retro-ocular capillary bed at 0 and 10 days of the experiment for hemoglobin determination. Body weights will be determined also at 0 and 10 days.

## 4. Analytical Procedures

Hemoglobin will be quantitated from duplicated samples of blood by colorimetry (Crosby et al., 1954). Hemoglobin Regeneration Efficiency (HRE) will be calculated for each animal as follows:

$$\text{Efficiency} = \frac{\text{mg HbFe (day 10)} - \text{Mg HbFe (day 0)}}{\text{mg Fe consumed days 0 to 10}} \times 100$$

Milligrams hemoglobin iron (mg Hb Fe) will be calculated at days 0 and 10 for individual rat knowing body weights, hemoglobin concentrations. Milligrams of iron consumed will be calculated multiplying the difference between food given and food spilled or refused (net food intake) by the analyzed iron value (Fe concentration) for that diet.

The data will be analyzed statistically by analysis of variance. When F is significant ( $P < 0.05$ ), least significant difference values will be calculated to identify differences among treatment means (Carma and Swanson, 1973).

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# Cost requirements for project

Salary (Research Associate)	100% time x 12 mo	\$12,000.00
	Benefit (.315)	3,780.00
Panel servant	40 hours	\$5.00/hr
		200.00
Animal care	100 hours	\$5.00/hr
		500.00
	Benefit	105.00
	subtotal	<u>\$16,585.00</u>
Animal (rats)	200 x \$3.25 each	650.00
	shipping and handling	180.00
Cheese	20 lb for rats, 30 lb for panel	
	50 lb x \$3.00	150.00
Milk		125.00
Iron sources	10 x \$40.00 each	400.00
Diets	200rat x 10g/d x 17d=34kg=75lb x 5.00	375.00
Laboratory animal research center for animal care		
	200rats x 17d x \$0.12/rat day	408.00
	subtotal	<u>\$2,288.00</u>
Glassware		
	Crucibles \$97.45/36	97.45
	Test tubes \$143.28/720	143.28
	Funnels \$30.36/12	30.36
Chemicals and acids		200.00
Panel costs		
	Ice cream cone 50 people x 6 x \$0.40	120.00
	Facilities	80.00
	Cups, evaluation forms, misc.	100.00
	subtotal	<u>\$771.00</u>
Computer		150.00
Photocopies		100.00
Publication		200.00
Attend meeting to present results		850.00
	subtotal	<u>\$1,300.00</u>
	Total costs:	<u>\$20,944.00</u>

## Project Title: Iron Fortification of Cheese Curd

**Personnel:** Arthur W. Mahoney, Professor, Nutrition and Food Sciences, Utah State University

Dejia Zhang, Research Associate, Nutrition and Food Sciences, Utah State University

**Funding Sources:** Western Dairy Food Research Center  
Utah Agricultural Experiment Station

## Objectives:

1. To evaluate effects of 12 months aging on quality of iron fortified cheeses prepared in the last few months of the current project.
2. To determine the iron binding characteristics of iron-casein, ferrisodium phosphate whey protein (FIP-WP) and  $\text{FeCl}_3$ -whey protein (Fe-WP) complexes prepared with different iron concentrations.
3. To determine the effects of iron fortification with 'optimized' iron-protein complexes on cheese quality.
4. To determine the effects of iron fortification on yogurt quality.
5. To determine the bioavailability of iron in the 'optimized' iron-protein complexes as well as yogurt and cheese fortified with them.

## Results:

### To objectives 1 and 3.

Effect of Iron Fortification on Quality of Cheddar Cheese II. Effect of Aging and Fluorescent Light on Pilot Scale Cheeses. (Manuscript in preparation)

Based on the success of iron fortification of Cheddar cheese at the laboratory level (about 1 kg) in a preliminary study, a medium scale experiment (about 12 kg) was conducted to confirm the results and to obtain more information. An unfortified control cheese and four cheeses fortified with Fe-casein, ferrisodium phosphate-whey protein (FIP-WP), Fe-whey protein (Fe-WP), or  $\text{FeCl}_3$  were made. Iron levels and recoveries were 40, 39, 39 and 42 mg/kg, and 69, 68, 65 and 70% for cheeses fortified with Fe-casein, FIP-WP, Fe-WP and  $\text{FeCl}_3$ , respectively. Cheese yield ranged from 9.1 to 9.7%. Iron fortification did not affect cheese moisture, fat, protein, Na and Ca contents.

TBA number and trained taste panel scores are listed in Table 1. TBA number of iron fortified cheeses and control cheese were undetectable up to 1 mo aging and remained low thereafter through 12 mo of aging. TBA number was not affected ( $P>0.05$ ) by iron fortification. Trained taste panelists judged low oxidized flavor for control and Fe-WP fortified cheeses at 15 d of aging and did not distinguish any differences ( $P>0.05$ ) for oxidized off-flavor among the cheeses thereafter. Oxidized off-flavor did not increase ( $P>0.05$ ) with aging up to 12 mo. Trained taste

panelists judged better cheese flavor for cheeses fortified with FIP-WP, FeCl<sub>3</sub> or Fe-WP at 1 mo of aging, for cheese fortified with Fe-casein at 9 mo, and did not distinguish differences in cheese flavor at 15 d, 4 mo, 7 mo or 12 mo.

Hedonic scores from the open panel of 66 lay subjects were evaluated. The judges scored all cheeses above average on the preference scale. The panelists did not detect differences ( $P>0.05$ ) in texture among the cheeses. They preferred control cheese and the cheese fortified with Fe-casein over other cheeses for cheese flavor and overall quality.

TBA values of cheeses up to 28 d intensive fluorescent light exposure were determined. Cheeses aged for 6 mo were cut into small blocks, 14 x 10 x 2.5 cm and re-vacuum sealed in polyethylene bags, 18 x 30 cm, of 0.06 mm thickness. Sealed cheese blocks were placed in a ventilated cool room at 10°C (50°F) under fluorescent lighting which was kept on 24 h per day at a light intensity of about 1280 lux. TBA values of cheese (about 2 mm thick of surface towards lights) were determined 0 d, 3 d, 7 d, 14 d and 28 d of light exposure. Intensive light exposure slightly increased TBA number after 7 d which did not differ significantly among the cheeses. TBA numbers of the first inner 1 mm layer of cheese were higher than the second 1 mm layer in all cheeses after 28 d of light exposure, indicating that light exposure does influence the oxidation process of cheese. However, the TBA number of the inner 1 mm cheese layer was not different from cheeses stored in the dark. Compared with unfortified cheese, fortifying iron into cheese with different iron sources did not change TBA number under this intensive light exposure.

#### To objective 5.

Bioavailability of Iron-milk-protein Complexes and Fortified Cheddar Cheese. (Manuscript submitted)

Cheddar cheese was fortified with FeCl<sub>3</sub> or iron-casein, FIP-WP and Fe-WP complexes. Bioavailability of iron from iron fortification sources and fortified cheeses was determined by hemoglobin regeneration efficiency (HRE), a determination of percentage dietary iron incorporated into hemoglobin. Maximal iron bioavailability was measured in anemic weanling rats fed low-iron diets (about 22 mg iron/kg) for the iron fortification sources and fortified cheeses. Basal iron bioavailability was determined for iron fortification sources in normal adult rats fed high-iron diets (about 145 mg iron/kg) of iron density (32 mg iron/1000 kcal) found in some high-iron human diets.

HRE values of the anemic rats fed iron-fortified cheeses are listed in Table 2. More than two-thirds of the dietary iron was incorporated into Hb in anemic rats fed on iron-fortified cheese diets. There were no statistically significant differences ( $P>0.05$ ) in HRE among the cheese diets. The HRE values of the iron-protein complexes were similar whether they were mixed directly into diets or in fortified cheese and then mixed into diets ( $P>0.05$ ). The HRE values were the same for the FeSO<sub>4</sub> and FeCl<sub>3</sub> supplemented diets.

HRE values of adult rats fed high-iron diets (basal iron bioavailability) were much lower than those of anemic growing rats fed low-iron diets (maximal iron bioavailability) (6% versus 77%,  $P<0.01$ ). Iron status and dietary iron level strongly affected the iron bioavailability values for all iron sources. However, differences of bioavailability among the iron sources were not significant ( $P>0.05$ ) either in anemic rats fed low-iron or in normal rats fed high-iron diets (Table 2). The basal iron bioavailability values were very similar to iron bioavailability values for typical nonheme dietary iron sources reported for normal humans.

Feeding the high iron diets to adult rats for 14 d resulted in lower HRE than feeding for 10 d ( $P<0.05$ ); the rats had no further increase in Hb iron during the additional 4 d of feeding indicating that the rats regulate the absorption of iron from these iron fortification sources

well. Longer time determination is needed to further confirm the capability of rats to regulate iron absorption, information which will be necessary in the establishment of the safety of iron fortified cheese. It will be necessary to first establish the most suitable iron sources for cheese manufacture, however.

Objective 2 will be studied in a separate experiment started in July, 1989.

Objective 4 will be started in July, 1989. Instead of yogurt, process cheese fortified with iron will be studied.

This project has resulted in one published paper, one paper in-press, and one manuscript in preparation on iron-fortified Cheddar cheese. We anticipate that a fourth manuscript will be written on iron-fortified process cheese. Four papers have been presented at scientific meetings.

### **Impact of Research:**

Iron deficiency anemia is still a major worldwide nutritional problem. It is hard to increase dietary iron intake because dairy products, the major sources of dietary calcium which compose a large portion of diets, contain almost no iron. The people who consume high iron density diets (9 or more mg Fe/1000 kcal) consume the least dairy products while those with lower iron density diets consume more dairy products. Iron-fortified dairy products could be promoted for increasing the iron density of the low-iron diets as well as calcium and vitamin D contents thereby addressing two major nutritional concerns of women and children: i.e., iron deficiency (women, children under age 2 and elderly men) and osteoporosis (middle-aged women, growing children, elderly men).

Iron has not been fortified into dairy products because fortified iron causes oxidative damage. No trial on iron fortification of cheese has been reported. The results reported here confirmed that Cheddar cheese could be fortified with iron up to 12 months of aging without oxidative damage and iron fortification did not cause oxidative damage when the cheeses displayed under intensive fluorescent light exposure for 28 d. These results indicate the possibility that cheese could be fortified with iron in a large factory scale and iron fortified cheese could be kept in market condition without deterioration. They also indicate that other dairy products could be fortified with iron sources used in this study to avoid oxidative damage.

Iron bioavailability of fortified cheese is important information concerning the value of fortified iron. Although bioavailability of iron in cow's milk is reported to be lower than that of human and goat milks, iron fortified cheese had highly bioavailable iron and could provide sufficient iron to meet human needs.

Food consumption trends of US women has been changing from 1977 to 1985 as an impact of nutritional messages. Consumption of lower-fat milks increased and whole milk decreased over the last decade. The percentage of women consuming higher-fat cheeses has also increased with no change observed in per capita consumption. These changes may be impacted by the nutritional messages, such as low fat and high fiber in diet may decrease the risk of cancer, and high calcium may lower the risk of osteoporosis and hypertension. However, when iron deficiency is the immediate nutritional concern of the people, the consumption of dairy products and other low-iron foods may decrease. Iron fortified cheese may provide the US population a healthier and more nutritional food and may add an extra product to counter for the choice of people concerned with their iron intakes.

Although it is difficult for me to estimate the economic impact of iron-fortification to the dairy industry, iron-fortified cheese could become a popular product in the fast food industry that serves major amounts of foods to growing children, especially adolescents and teenagers. Iron fortified dairy products could become popular with women who are frequently diagnosed with iron deficiency anemia and are encouraged to consume iron-rich diets during pregnancy. Typically, there is a dietary dilemma between balancing the diet meet the Recommended Daily Allowance (RDA) for calcium from dairy products, which are practically devoid of iron, and balancing the diet to meet the RDA for iron, which is high (about 9mg Fe/1000 kcal) for women and children. Fortifying dairy products at about 10 mg Fe/1000 kcal would eliminate this dilemma and dieticians would not have to restrict the level of dairy products in diets on this basis. Depending on the level and nature of promotion, iron-fortified dairy products could make up several percentage points of the cheese, cottage cheese and yogurt produced.

#### **Publications:**

\*Zhang, D., and A.W. Mahoney. 1989. Effect of iron fortification on quality of Cheddar cheese. *J. Dairy Sci.* 72:322-332.

\*Zhang, D., D.G. Hendricks and A.W. Mahoney. 1989. Bioavailability of total iron from meat, spinach (*Spinacea oleracea* L.) and meat/spinach mixtures by anaemic and non-anaemic rats. *Br. J. Nutr.* 61:331-343.

\*Zhang, D., and A.W. Mahoney. 1989. Bioavailability of iron-milk protein complexes fortified in Cheddar Cheese. *J. Dairy Sci.* (In press)

\*Zhang, D., and A.W. Mahoney. Effect of iron fortification on quality of Cheddar cheese. II. Effects of aging and fluorescent light on pilot scale cheeses. *J. Dairy Sci.* (In preparation)

\*Mahoney, A.W., and D. Zhang. 1988. Progress toward fortifying cheese with iron. Proceedings of the 8th Biennial Cheese Conference, Utah State University, Logan, UT.



TABLE 1. TBA values and trained taste panel scores of iron-fortified cheese

Iron Source	Control	FIP-WP	Fe-casein	Fe-WP	FeCl <sub>3</sub>	LSD <sup>1</sup>
Cheese Fe, µg/g	1	39	40	39	42	---
TBA Number						
7 days	0.00	0.00	0.00	0.00	0.00	---
1 month	0.00	0.00	0.00	0.00	0.00	---
4 months	0.05	0.16	0.16	0.12	0.18	---
7 months	0.00	0.03	0.00	0.03	0.05	---
9 months	0.02	0.03	0.10	0.06	0.07	---
12 months	0.03	0.08	0.07	0.07	0.07	---
Taste panel score <sup>2</sup>						
Oxidized flavor						
15 days	1.4a	4.2c	3.6c	2.0ab	3.1bc	1.5
1 month	3.4	3.0	4.8	4.0	2.8	NS
4 months	3.3	3.8	4.2	4.8	4.5	NS
7 months	3.2	3.0	3.8	5.2	5.3	NS
9 months	2.9	3.6	2.4	4.0	3.5	NS
12 months	3.1	3.4	5.6	5.7	4.3	NS
Cheese flavor						
15 days	6.7	4.8	6.0	5.6	5.5	NS
1 month	5.9bc	7.7a	5.5c	6.4abc	7.0ab	1.4
4 months	6.0	4.9	4.4	3.8	4.7	NS
7 months	5.9	6.1	5.8	5.3	4.8	NS
9 months	5.4bc	4.4c	7.2a	4.8bc	5.6b	1.2
12 months	6.5	6.5	5.6	4.0	6.2	NS

a,b,c Means with the same superscripts are not significantly different.

<sup>1</sup> Least significant difference values were calculated when F was larger than F<sub>0.05</sub> (P < 0.05).

<sup>2</sup> Taste panel scores were set from 1 to 10. For oxidized flavor, the higher score indicated a stronger flavor. For cheese flavor, the higher score indicated a better quality.

TABLE 2. Iron bioavailability determined by hemoglobin regeneration efficiency in rats<sup>1</sup>

	FeCl <sub>3</sub>	Fe-casein	FIP-WP	Fe-WP	FeSO <sub>4</sub>
<i>Iron fortified cheese</i>					
Rats' iron status	D	D	D	D	
Rats' growing stage	G	G	G	G	
Iron intake	2.05	1.98	2.00	2.09	
Repletion period	10	10	10	10	
HRE	75	66	74	67	
<i>Iron fortification source</i>					
Rats' iron status	D	D	D	D	D
Rats' growing stage	G	G	G	G	G
Iron intake	2.30	2.05	2.15	2.33	2.22
Repletion period	10	10	10	10	10
HRE	85	71	73	72	85
-----					
Rats' iron status	N	N	N	N	N
Rats' growing stage	A	A	A	A	A
Iron intake	15.31	14.33	15.07	15.11	15.37
Repletion period	10	10	10	10	10
HRE	5	8	6	7	5
-----					
Iron intake	21.04	21.00	20.55	20.96	21.11
Repletion period	14	14	14	14	14
HRE	4	4	3	3	5

<sup>1</sup> Abbreviations: D=iron-deficient; G=growing; N=normal; A=adult; HRE= hemoglobin regeneration efficiency.

**Project Title:** Iron Fortification of Cheese Curd

**Personnel:** Arthur W. Mahoney, Dept. of Nutrition and Food Sciences, Utah State University

Dejia Zhang, Nutrition and Food Sciences, Utah State University

**Funding Sources:** Western Dairy Food Research Center  
Utah Agriculture Experiment Station

### Objectives:

1. To evaluate effects of 12 months aging on quality of iron fortified cheeses prepared in the last few months of the current project.
2. To determine the iron binding characteristics of iron-casein, ferripolyphosphate whey protein (FIP-WP) and Fe-whey protein (Fe-WP) complexes prepared with different iron concentrations.
3. To determine the effects of iron fortification with 'optimized' iron-protein complexes on cheese quality.
4. To determine the effects of iron fortification on the quality of process cheddar cheese.
5. To determine the bioavailability of iron in the 'optimized' iron-protein complexes as well as cheese fortified with them.

### Results:

#### To objective 4

*Iron Fortification of Process Cheddar Cheese* (Manuscript submitted to J. Dairy Sci.)  
Four batches of iron-fortified process Cheddar cheese were prepared using different iron sources, viz., Fe-casein, Fe-whey protein and  $\text{FeCl}_3$  for fortification. All process Cheddar cheeses fortified with iron in this study had their iron level increased to about 40 mg/kg (Tables 2-5). One hundred percent of fortified iron was kept in the cheese which is an advantage compared to fortification of milk for making natural Cheddar cheese. Moisture and fat contents in all cheeses were within the standards for process cheese, except the cheeses in batch 4 which had slightly lower fat contents (Tables 2-5). Formulation and composition of process Cheddar cheese is given in Table 1.

Oxidized off-flavor was not increased in the fortified process cheeses as compared with the control unfortified cheese ( $P > .05$ ) (Tables 2-5). Also, cheese flavor of the fortified process cheese was similar ( $p > .05$ ) to the control cheese. TBA scores of all cheeses were low during all time periods (10 - 90 d) determined. When the values of the batches were pooled together, average TBA scores were .07, .11, .11, and .10 for control, Fe-casein, Fe-WP and  $\text{FeCl}_3$  fortified cheeses, respectively. The differences in average TBA scores among the groups were marginally significant ( $P = .049$ ) with the

control cheese having a slightly lower TBA score than Fe-casein and Fe-WP cheeses (LSD=.032). TBA scores of the cheeses did not increase with the time of storage. The open taste panel of 94 volunteer lay subjects did not detect significant differences in texture and flavor among the iron fortified and control cheeses ( $P>.05$ ) (Table 6). This is consistent with the evaluations of expert taste panelists (Table 5) for this batch of cheeses.

In this study, several factors were varied among the different batches of process cheese, but none of them affected the quality of fortified vs. unfortified control cheese. First, the source of cheese and the proportion of aged cheese in the formulations were varied. The cheeses used in batch 4 were from a different source than the other three batches and the proportion of young, medium and aged cheese in batch 4 also differed. Second, 10% less cream and about twice as much whey powder was used in batch 4 as compared with the other three batches. Third, salt was reduced to 2/3 in batches 2 and 3, and to about 1/2 in batch 4 as compared with batch 1. Fourth, taco sauce was not included in the batches 2, 3, and 4. All these changes were based upon the flavor preference, the standards for process cheese and consideration of determining more variables such as the cheese source, proportion of various aged cheeses and addition of taco source. However, by analysis of variance, the effect of iron fortification on the cheese quality was not statistically significant among these batches of process cheese. Therefore, it appears that iron fortification of process cheese is very flexible to variations in formulation and cheese sources.

The TBA and taste panel scores of the process cheese fortified with  $\text{FeCl}_3$  were similar to those of the cheese fortified with Fe-casein or Fe-WP, indicating that pre-binding of iron to proteins is not necessary. Iron from ferric salts and small molecular complexes, such as  $\text{FeCl}_3$  and  $\text{Fe}^{3+}$ -fructose, readily binds to proteins forming iron-protein complexes.

Iron fortification did not affect the quality of Cheddar cheese in our previous study and of process cheese in this study although some differences exist between these two cheeses. For Cheddar cheese, milk coagulation is involved, heating is at low temperature, pH is low, and microbial organisms from the starter culture are still growing during the aging period. In contrast, for process cheese, a relatively high temperature is applied, pH is high, and other ingredients are added to the cheese. However, they both have high protein contents which may act as a chelator of iron. This may be the main reason that low lipid peroxidation was observed in both kinds of iron fortified cheeses. Another reason for low lipid peroxidation may be the saturation of the fat. Milk fat contains mostly saturated fatty acids, 30% of the fatty acids are unsaturated of which about 3% are polyunsaturated. Also, for lipid peroxidation to occur, both  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  are required with maximal rates of lipid peroxidation at the ratio of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  being approximately one. However, it is unlikely that iron bound to milk protein is free to change its oxidation state at the pH of cheese. Therefore, the conditions are favorable for iron fortification of cheese products.

## Impact of Research:

Iron deficiency anemia is still a major world wide nutritional problem. It is hard to increase dietary iron intake because dairy products, the major sources of calcium which compose a large portion of diets, contain no iron. The people who consume high iron density diets (9 or more mg Fe/1000kcal) consume the least dairy products while

those with lower iron density diets consume more dairy products. Iron fortified dairy products could be promoted for increasing the iron density of the low-iron diets as well as calcium and vitamin D contents thereby addressing two major nutritional concerns of women and children: i.e., iron deficiency (women, children under age 2 and elderly men) and osteoporosis (middle-aged women, growing children and elderly men).

About two billion pounds of process cheese and cheese foods are produced each year in the U.S.. Young children and teenagers enjoy process cheese, making iron fortified process cheese more meaningful to target this population in prevention of iron deficiency. Using the iron fortification level of 40 mg/kg cheese, and if all process cheese and cheese foods were fortified with iron, it would provide 36 billion mg more dietary iron to U.S. consumers each year. Overall, an average of .5 mg extra dietary iron per person per day would be provided from process cheese. Fortified process cheese would be expected to contribute relatively more benefit to children and teenagers who are at risk of iron deficiency, because they eat more process cheese than other segments of the populations. Iron fortification of process cheese improves this product nutritionally from almost no iron to an iron-rich food, 11 mg/1000 kcal.

#### **Publications:**

Zhang, D. & A.W. Mahoney. 1989. Effect of iron fortification on quality of Cheddar cheese. *J. Dairy Sci.* 72:322.

Zhang, D. & A.W. Mahoney. 1989. Bioavailability of iron-milk-protein complexes and fortified Cheddar cheese. *J. Dairy Sci.* 72:2845.

Zhang, D and A.W. Mahoney. 1990. Effect of iron fortification on quality of Cheddar cheese. II. Effects of aging and fluorescent light on pilot scale cheeses. *J. Dairy Sci.* In press.

Zhang, D. & A. W. Mahoney. 1990. Iron Fortification of Process Cheddar Cheese. Submitted to *J. Dairy Sci.*

TABLE 1. Formulation of process Cheddar cheese (g)

Ingredients	Batch 1	Batches 2 & 3	Batch 4
Cheddar	1273	1273	1272
Young (40%)	508	508	424
Medium (40%)	508	508	424
Aged (40%)	257	257	424
80% cream	218	218	200
Water	164	175	200
Whey powder	73	73	140
Sodium citrate	36	36	36
DSP <sup>1</sup>	18	18	18
NaCl	18	12	10
Sorbate	2.3	2.3	2.3
Taco sauce	18	---	---
TOTAL	1820.3	1807.3	1878.3

<sup>1</sup> DSP stands for disodium phosphate.

TABLE 2. Iron contents and qualities of iron fortified process cheddar cheese, batch 1

Process cheese a=.05	Control	Fe-casein	Fe-WP	FeCl <sub>3</sub>	LSD,
Iron content, mg/kg	5	45	47	50	---
Moisture, %	38.4	38.5	39.0	39.2	---
Fat, %	34.2	34.3	32.7	32.7	---
TBA number					
10 d	.05	.14	.09	.07	---
30 d	.06	.12	.20	.18	---
90 d	.05	.12	.09	.11	---
Taste panel score <sup>1</sup>					
Oxidized off-flavor					
10 d (7) <sup>2</sup>	2.3	2.1	2.2	2.4	NS
30 d (8)	2.4	2.0	2.1	2.6	NS
90 d (10)	2.9	3.3	2.8	3.4	NS
Cheese flavor					
10 d (7)	7.0	6.6	6.1	6.7	NS
30 d (8)	5.6	5.8	5.9	5.5	NS
90 d (10)	5.7	5.8	6.0	5.6	NS

<sup>1</sup> Taste panel scores were set from 1 to 10. For oxidized flavor, the higher score indicated a stronger flavor. For cheese flavor, the higher score indicated a better quality.

<sup>2</sup> The value in the parenthesis is the number of the panelists.

TABLE 3. Iron contents and qualities of iron fortified process cheddar cheese, batch 2

Process cheese	Control LSD, $\alpha=.05$	Fe-casein	Fe-WP	FeCl <sub>3</sub>	
Iron content, mg/kg	4	35	40	44	---
Moisture, %	38.1	36.5	38.6	38.0	---
Fat, %	34.4	34.6	34.3	33.8	---
TBA number					
10 d	.07	.16	.12	.12	---
30 d	.14	.14	.16	.12	---
90 d	.06	.08	.09	.06	---
Taste panel score <sup>1</sup>					
Oxidized off-flavor					
10 d (8) <sup>2</sup>	2.0	2.4	2.4	2.1	NS
30 d (8)	2.4	2.2	2.3	2.5	NS
90 d (8)	1.6	1.8	1.8	1.8	NS
Cheese flavor					
10 d (8)	6.4	6.0	6.2	6.4	NS
30 d (8)	6.6	6.7	6.7	7.0	NS
90 d (8)	5.8	6.1	6.6	6.2	NS

<sup>1</sup> Taste panel scores were set from 1 to 10. For oxidized flavor, the higher score indicated a stronger flavor. For cheese flavor, the higher score indicated a better quality.

<sup>2</sup> The value in the parenthesis is the number of the panelists.



TABLE 4. Iron contents and qualities of iron fortified process cheddar cheese, batch 3

Process cheese	Control LSD, $\alpha=.05$	Fe-casein	Fe-WP	FeCl <sub>3</sub>	
Iron content, mg/kg	2	41	39	39	---
Moisture, %	37.3	37.2	37.2	37.3	---
Fat, %	32.2	33.6	33.6	34.6	---
TBA number					
10 d	.05	.07	.06	.11	---
30 d	.09	.10	.09	.09	---
90 d	.08	.10	.12	.08	---
Taste panel score <sup>1</sup>					
Oxidized off-flavor					
10 d (8) <sup>2</sup>	2.8	2.4	3.0	3.1	NS
30 d (9)	2.3	2.1	1.9	1.9	NS
90 d (8)	2.1	2.6	2.2	1.9	NS
Cheese flavor					
10 d (8)	7.2	7.1	6.6	6.4	NS
30 d (9)	6.4	7.0	6.1	6.8	NS
90 d (8)	6.5	5.9	5.5	6.6	NS

<sup>1</sup> Taste panel scores were set from 1 to 10. For oxidized flavor, the higher score indicated a stronger flavor. For cheese flavor, the higher score indicated a better quality.

<sup>2</sup> The value in the parenthesis is the number of the panelists.

TABLE 5. Iron contents and qualities of iron fortified process cheddar cheese, batch 4

Process cheese	Control LSD, $\alpha=.05$	Fe-casein	Fe-WP	FeCl <sub>3</sub>	
Iron content, mg/kg	2	43	45	42	---
Moisture, %	38.5	38.6	39.3	39.6	---
Fat, %	29.5	29.6	30.0	29.4	---
TBA number					
15 d	.08	.09	.08	.06	---
90 d	.05	.08	.07	.06	---
Taste panel score <sup>1</sup>					
Oxidized off-flavor					
15 d (9) <sup>2</sup>	2.4	2.5	2.5	1.8	NS
90 d (8)	3.2	3.3	2.9	3.4	NS
Cheese flavor					
15 d (9)	6.4	5.6	6.0	5.9	NS
90 d (8)	5.8	6.1	6.2	6.7	NS

<sup>1</sup> Taste panel scores were set from 1 to 10. For oxidized flavor, the higher score indicated a stronger flavor. For cheese flavor, the higher score indicated a better quality.

<sup>2</sup> The value in the parenthesis is the number of the panelists.

TABLE 6. Open taste panel scores for iron-fortified cheese<sup>1</sup>, batch 4

	Control	Fe-casein	Fe-WP	FeCl <sub>3</sub>	LSD <sup>2</sup>
Texture	6.3	6.1	5.9	6.0	NS
Flavor	6.2	6.0	5.6	6.0	NS
Overall	6.1	5.9	5.7	6.0	NS

<sup>1</sup> Taste panel scores were hedonic scores set from 1 to 10 for which 1 was "dislike extremely" and 10 was "like extremely." Each value is a mean of 94 volunteer lay subjects.

<sup>2</sup> LSD means least significant difference values which would be calculated when F was larger than  $F_{.05}$  ( $P < .05$ ). NS means not statistically significant ( $P > .05$ ).

**Project Title:**

Application of Fourier Transform Infrared Technology to Milk and Dairy Products

**Personnel:**

Rodney J. Brown, Professor, Nutrition and Food Sciences, Utah State University;  
Ivan Mendenhall, Senior Research Technician, Nutrition and Food Sciences, Utah State University

**Funding Sources:**

Western Dairy Foods Research Center; Utah Agricultural Experiment Station

**Objectives:**

This project has a long series of specific objectives that lead to the ability to rapidly measure fat, protein, lactose, moisture, and fat saturation level in milk and dairy products. These specific objectives must be accomplished in a logical order:

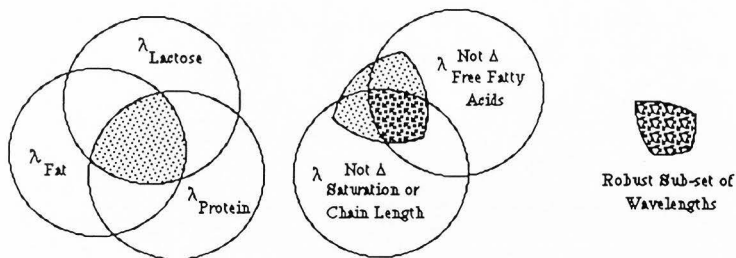
1. Find a set of wavelengths in the infrared spectrum that respond to changes in fat concentration.
2. Find a set of wavelengths in the infrared spectrum that respond to changes in protein concentration.
3. Find a set of wavelengths in the infrared spectrum that respond to changes in lactose concentration.
4. Find a set of wavelengths in the infrared spectrum that do not respond to changes in saturation level and chain length of fatty acids.
5. Find a set of wavelengths in the infrared spectrum that do not respond to changes in free fatty acid levels
6. Combine these (1-5) to make a robust set of wavelengths common to all constraints.
7. From the total infrared spectrum of milk, determine the individual spectra of:
  - ☐ Milk fat
  - ☐ Milk protein
  - ☐ Milk lactose
8. Find a set of wavelengths common to milk fat, milk protein, and milk lactose.
9. Find a set of wavelengths common to the milk components (8) and to the robust set (6).

10. Statistically calibrate for testing samples of unknown composition using only this set of wavelengths (9) and milk samples chemically tested for fat, protein, lactose, and moisture. (Less than 1600  $\text{cm}^{-1}$  wavenumber should be used if possible.)
11. Establish sample preparation procedures and calibrate to test dairy products other than milk.
12. Calibrate, using wavenumbers greater than 2700  $\text{cm}^{-1}$ , to determine saturation level of the fat in dairy products (especially cheese).

## Results:

The total infrared spectrum of milk has been divided into the individual spectra of fat, protein, and lactose. These three separate spectra were found by recording the spectra of whole milk, skim milk and a lactose solution with the same lactose concentration as the milk. The fat spectra was found by subtracting the skim milk spectra from the milk spectra, the protein spectra by subtracting the lactose solution spectra from the skim milk spectrum, and the lactose spectrum was measured directly. One additional spectrum, that of the milk salts, will be found by subtracting the fat, protein and lactose spectra from the milk spectrum.

Sets of wavelengths in the infrared range that respond to changes in fat, protein and lactose concentrations have been found. We are now looking for sets of wavelengths that do not respond to changes in saturation level or chain lengths of fatty acids. These will be statistically analyzed to see which wavelengths are least affected by variations in saturation level and chain length. A set of wavelengths that do not respond to changes in free fatty acid levels will next be found. Results of the



experiments to this point will be combined into a set of wavelengths that respond to changes in fat, protein, and lactose but are not affected by type of fatty acid or degree of lipolysis.

Milk samples chemically tested for fat, protein, lactose, and moisture have been used to make preliminary statistical calibrations for testing samples of unknown composition. The mathematical procedures work. When the experiments described above are completed, calibration will be done using only wavelengths meeting the requirements they impose.

A series of procedures will be developed for preparation of dairy product samples to be tested. Concentrations of components will be near the ideal range for testing. Solvents will be selected for their ability to solubilize the products and absence of absorbance by the solvents at the wavelengths used. The first dairy food to be considered will be cheese. The next likely product for analysis will be butter-margarine blends.

### **Impact of Research:**

This project will provide an improved method for measuring fat, protein, lactose and water content in dairy products. Most methods that have been adapted from laboratory configurations to industrial applications are based on very simple, usually old, technology (Brown, 1986). This is true of instruments used for testing milk and dairy products. The instruments we use in dairy testing were state of the art instruments thirty years ago (Hirschfield, et al., 1984). A fast method for detecting non-dairy ingredients, particularly fat, in products labeled or sold as dairy products is needed. Adulterated products sold as dairy products replace real dairy products.

For many years fixed filter spectroscopy has been the most common method for measuring fat, protein and lactose in milk (Goulden, 1956, 1961, 1964). Improvements over the years have not overcome the limitations imposed by the small number of filter bands available in such instruments (Grappin, 1984; Sjaunja and

Anderson, 1985). Nor have they overcome interference by changing levels of saturation in milk fat from one sample to the next or the effects of lipolysis on milk fat (Brown, 1986).

Fourier Transform Infrared (FTIR) has many advantages over fixed filter methods. Fixed filter instruments are limited to a few (usually not more than four) pairs of filters. These must be rotated into the light path for measurements to be made. With FTIR, measurements at hundreds of different wavelengths could be made almost instantaneously. FTIR measurements are very narrow bands of the spectrum instead of the broad bands of filter measurements. With additional wavelengths available, measurements can be made more accurately. The large number of measurements possible in a short time allows much more powerful data processing methods to be used. Any number or combination of readings can be used to measure any component. Handling of this large amount of data is no longer a problem with computers built into all new instruments. An FTIR instrument can consider variables such as saturation level of the fat, lipolysis of fat, etc. so they do not interfere with accurate measurements. Calibration of the instruments will be less frequent.

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- Brown, R.J., 1986, Modern methods of analysis of milk and milk products: Rapid determination of main components. pp. 507 *In* Milk the Vital Force. Reidel Publ. Co., Dordrecht, The Netherlands.
- Goulden, J.D.S., 1956, J. Sci Food Agric. September 7:609.
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- Sjaunja, L.-O. and I. Anderson, 1985, Acta Agric. Scand. 35:345.
- Warner, I.M., E.R. Davidson and G.D. Christian, 1977, Anal. Chem. 49:2155.

**Project Title:**           **Application of Fourier Transform Infrared Technology to Infrared Technology to Milk and Dairy Products**

**Personnel:**           Rodney J. Brown, Professor, Nutrition and Food Sciences, Utah State University  
  
                  Ivan Mendenhall, Senior Research Technician, Nutrition and Food Sciences, Utah State University.

**Funding Sources:**    Western Dairy Foods Research Center  
                              Utah Agricultural Experiment Station

**Objectives:**

This project has a long series of specific objectives that lead to the ability to rapidly measure fat, protein, lactose, moisture, and fat saturation level in milk and dairy products. These specific objectives must be accomplished in a logical order:

1. Find a set of wavelengths in the infrared spectrum that respond to changes in fat concentration.
2. Find a set of wavelengths in the infrared spectrum that respond to changes in protein concentration.
3. Find a set of wavelengths in the infrared spectrum that respond to changes in lactose concentration.
4. Find a set of wavelengths in the infrared spectrum that do not respond to changes in saturation level and chain length of fatty acids.
5. Find a set of wavelengths in the infrared spectrum that do not respond to changes in free fatty acid levels.
6. Combine these (1-5) to make a robust set of wavelengths common to all constraints.
7. From the total infrared spectrum of milk, determine the individual spectra of:
  - ☐ Milk fat
  - ☐ Milk protein



### □ Milk lactose

8. Find a set of wavelengths common to milk fat, milk protein, and milk lactose.
9. Find a set of wavelengths common to the milk components (8) and to the robust set (6).
10. Statistically calibrate for testing samples of unknown composition using only this set of wavelengths (9) and milk samples chemically tested for fat, protein, lactose, and moisture. (Less than 1600  $\text{cm}^{-1}$  wavenumber should be used if possible.)
11. Establish sample preparation procedures and calibrate to test dairy products other than milk.
12. Calibrate, using wavenumbers greater than 2700  $\text{cm}^{-1}$ , to determine saturation level of the fat in dairy products (especially cheese).

### Results:

Infrared analysis of milk, developed in the 1960's, has played a key role in the technological development of the dairy industry. Dairy product manufacturers can obtain "real time" results on the fat, protein, and lactose levels in milk. This allows them to comply with standards of identity, maximize yields, and achieve desirable characteristics in the end product. The ability to economically and regularly monitor the levels of fat, protein, and solids in individual cow milk, allows milk producers to adjust feeding practices and breeding programs for optimal yield and quality.

Every instrumental method, however, has inherent limitations. The effects of fatty acid composition and lipolysis on infrared fat protein and lactose readings has been a source of concern and has been well documented in the literature. The objective of our research was to find wavelengths in the mid-infrared spectrum that respond to changes in fat, protein, and lactose concentration and yet have a low response to fatty acid variation and changes caused by lipolysis. A Fourier transform spectrometer provided us with high signal to noise ratio, highly reproducible, digitized spectra and computer capabilities needed for multivariate quantitative analysis.

To identify wavelengths which respond to changes in fat concentration, we prepared a series of milk samples where the fat level varied and all other components were held constant. The correlation coefficient for absorbance and fat concentration was calculated at each wavelength. A similar experiment was performed to determine wavelengths that linearly respond to changes in protein concentration. To determine wavelengths that linearly respond to changes in

lactose concentration, a series of lactose solutions were prepared there the lactose concentration varied from 1 to 5.5%.

Spectra of C18:0, C18:1, C18:2, and C18:3 fatty acids were measured. The standard deviation of absorbance at each wavelength was calculated. From this we can get a good idea of which wavelengths are most affected by changes in saturation of milk fat. Most obvious is the high variability at the fat B (3.4  $\mu$ ) and lactose (9.6  $\mu$ ) wavelengths. Spectra of C10:0, C12:0, C14:0, C16:0, and C18:0 fatty acids were measured. The standard deviation of absorbance at each wavelength was calculated. From this we can get a good idea of which wavelengths are most affected by changes in the chain-length of milk fat. Most obvious is the high variability at the fat A wavelength (5.75  $\mu$ ) and lactose (9.6  $\mu$ ) wavelengths. The effects of lipolysis on the spectrum were studied by adding lipase enzyme to 2% milk in the sample cell and collecting a spectrum every minute for one hour. A standard deviation was calculated at each wavelength. The most obvious variability occurs at the fat B (3.4  $\mu$ ) and protein (6.5  $\mu$ ) wavelengths.

We then selected wavelengths which were responsive to fat, protein, and lactose concentrations, and relatively unaffected by saturation, chain-length, and lipolysis. Nine calibration standards (each composed of milk from a separate herd) were purchased from a laboratory in southern California and the FTIR was calibrated using these standards and Partial Least Squares (PLS) statistics. The concentration of fat, protein, and lactose in these samples was then predicted using the generated calibration equations. The standard deviations of difference between chemical and predicted values lie close to the AOAC recommended SD of .06%. This data was obtained with no homogenization or temperature control. A liquid ATR cell was used and 64 scans at 4  $\text{cm}^{-1}$  resolution were averaged to obtain each sample spectrum.

### **Impact of Research:**

This project will provide an improved method for measuring fat, protein, lactose and water content in dairy products. Most methods that have been adapted from laboratory configurations to industrial applications are based on very simple, usually old, technology (Brown, 1986). This is true of instruments used for testing milk and dairy products. The instruments we use in dairy testing were state of the art instruments thirty years ago (Hirschfield, et al., 1984). A fast method for detecting non-dairy ingredients, particularly fat, in products labeled or sold as dairy products is needed. Adulterated products sold as dairy products replace real dairy products.

For many years fixed filter spectroscopy has been the most common method for measuring fat, protein and lactose in milk (Goulden, 1956, 1961, 1964). Improvements over the years have not overcome the limitations imposed by the small number of filter bands available in such instruments (Grappin, 1984; Sjaunja and Anderson, 1985). Nor have they overcome interference by changing levels of saturation in milk fat from one sample to the

next or the effects of lipolysis on milk fat (Brown, 1986).

Fourier Transform Infrared (FTIR) has many advantages over fixed filter methods. Fixed filter instruments are limited to a few (usually not more than four) pairs of filters. These must be rotated into the light path for measurements to be made. With FTIR, measurements at hundreds of different wavelengths could be made almost instantaneously. FTIR measurements are very narrow bands of the spectrum instead of the broad bands of filter measurements. With additional wavelengths available, measurements can be made more accurately. The large number of measurements possible in a short time allows much more powerful data processing methods to be used. Any number or combination of readings can be used to measure any component. Handling of this large amount of data is no longer a problem with computers built into all new instruments. An FTIR instrument can consider variables such as saturation level of the fat, lipolysis of fat, etc. so they do not interfere with accurate measurements. Calibration of the instruments will be less frequent.

### References:

- Brown, R.J., 1986, Modern methods of analysis of milk products: Rapid determination of main components. pp. 507 In *Milk the Vital Force*. Reidel Publ. Co., Dordrecht, The Netherlands.
- Goulden, J.D.S., 1956, J. Sci. Food Agric. September 7:609.
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- Warner, I.M., E.R. Davidson and G.D. Christian, 1977, Anal. Chem. 49:2155.

### Abstracts:

- Mendenhall, I.V., R.J. Brown, and R. Grappin. 1990. Alternative wavelengths for infrared measurement of fat, proteins, and lactose in milk. J. Dairy Sci. 73 (Suppl. 1):92.

**Project Title:** Estimation of Individual Milk Proteins and Genetic Variants by Multicomponent Analysis of Amino Acid Profiles

**Personnel:** Rodney J. Brown, Professor, Nutrition and Food Sciences, Utah State University

Marie Walsh, Graduate Student, Nutrition and Food Sciences, Utah State University

Susan Collinge, Research Associate, Nutrition and Food Sciences, Utah State University

Carol Hollar, Graduate Student, Nutrition and Food Sciences, Utah State University

**Funding Sources:** Western Dairy Foods Research Center  
Utah Agricultural Experiment Station  
USDA-ARS National Needs Graduate Fellowship Program

### Objectives:

The goal is to determine proportions of specific milk proteins, groups of proteins (such as whey protein or casein), and genetic variants of proteins in milk and other dairy products from information contained in a single amino acid analysis of the sample. The overall objective will be met by completing the following specific objectives:

1. Determine concentrations of groups of proteins in milk such as caseins or whey protein using amino acid analysis.
2. Determine concentrations of specific milk proteins: aS1, aS2, B, and K-Caseins, a-lactalbumin, B-lactoglobulin, and bovine serum albumin using amino acid analysis.
3. Separate genetic variants of specific milk proteins, and use amino acid analysis to quantify individual variants in a protein mixture.
4. Use techniques developed in 1-3 to analyze milk and other dairy products.
5. Determine mathematical procedures to obtain the most accurate and reproducible methods for estimating milk protein concentrations.

### Results:

The basis of this method is the knowledge that each class of milk protein has a different amino acid composition. Five pure milk proteins were prepared and analyzed by amino acid analysis.

They were  $\alpha$ -,  $\beta$ -, and  $\kappa$ -casein,  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin. Five different mixtures with varying ratios of these five proteins were made and analyzed for amino acid composition. Concentrations of individual proteins in each of these mixtures were estimated (Walsh and Brown, 1988a, 1988b).

Milk samples that have been characterized for specific milk proteins and genetic variants have been obtained from Dr. Juan Medrano at University of California, Davis (Medrano and Sharrow, 1989). Information obtained from amino acid analysis of these proteins will be used to determine proportions of specific proteins and genetic variants. Individual proteins will be quantified and compared with estimates from amino acid analysis.

### Impact of Research:

The overall goal of our dairy products research program is to develop new dairy products by using new manufacturing processes. To do this, we must be able to measure the individual components in milk without separating them from milk. Methods available to determine casein and whey protein concentrations in milk require precipitation of casein followed by protein measurement of both precipitate and whey. Measurement of specific milk proteins, including genetic variants, calls for further separation by quantitative electrophoresis and protein determination of each fraction (Basch et al. 1985). These procedures are time consuming, expensive and susceptible to errors.

The goal of this project is to determine proportions of specific milk proteins (down to the level of specific genetic variants) or groups of proteins in milk and other dairy products from the information contained in a single amino acid analysis of a sample. Relative proportions of milk proteins found in traditional dairy products are subject to change as ultrafiltration and other new processes are used in their manufacture. To use these emerging manufacturing processes to produce entirely new products without the information that will be made available when this project is completed is nearly impossible. Many areas of research will also be facilitated by the results of this research. We will be able to follow milk protein composition through lactation periods of individual cows (or other species), correlate content of each of the milk proteins with coagulation properties during cheese making, make artificial infant formula that more closely matches mothers' milk, etc.

### References:

- Basch, J.J., F.W. Douglas, Jr., L.G. Procino, V.H. Holsinger, and H.M. Farrell, Jr. 1985. Quantitation of caseins and whey Proteins of processed milks and whey protein concentrates, application of gel electrophoresis, and comparison with Harland-Ashworth procedure. *J. Dairy Sci* 68:23-31.
- Medrano, J.F. and L. Sharrow, 1989, Milk protein typing of bovine mammary gland tissue used to generate a cDNA library. Submitted for Publication.
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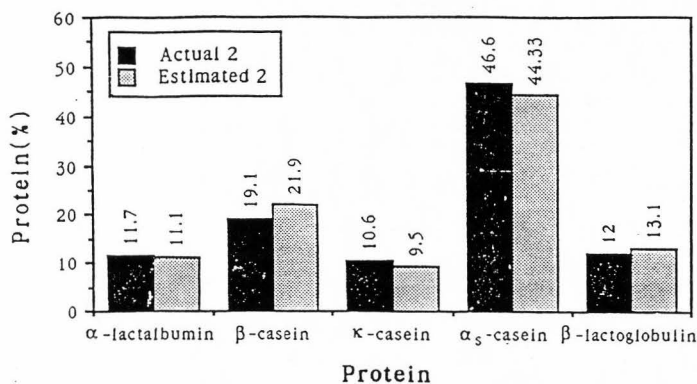


Figure 1. Concentrations of milk proteins in a mixture based on amino acid analysis estimates and measured values.

**Project Title:** Estimation of Individual Milk Proteins and Genetic Variants by Multicomponent Analysis of Amino Acid Profiles

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Carol Hollar, Graduate Student, Nutrition and Food Sciences, Utah State University

**Funding Sources:** Western Dairy Foods Research Center  
Utah Agricultural Experiment Station

**Objectives:**

The goal is to determine proportions of specific milk proteins, groups of proteins (such as whey protein or casein), and genetic variants of proteins in milk and other dairy products from information contained in a single amino acid analysis of the sample. The overall objective will be met by completing the following specific objectives:

1. Determine concentrations of groups of proteins in milk such as caseins or whey protein using amino acid analysis.
2. Determine concentrations of specific milk proteins:  $\alpha_{s1}$ ,  $\alpha_{s2}$ ,  $\beta$ , and  $\kappa$ -caseins,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, bovine serum albumin using amino acid analysis.
3. Separate genetic variants of specific milk proteins, and use amino acid analysis to quantify individual variants in a protein mixture.
4. Use techniques developed in 1-3 to analyze milk and other dairy products.
5. Determine mathematical procedures to obtain the most accurate and reproducible methods for estimating milk protein concentrations.

## Results:

Lyophilized milk samples of known genetic variant composition were obtained from Dr. Juan Medrano at the University of California, Davis. Samples were originally typed by the procedure of Medrano and Scharrow. They were later retyped using the procedure of Van Eenennaam and Medrano and several of the  $\kappa$ -casein variant designations were changed.

Samples for amino acid analysis were hydrolyzed in 6 N HCl for 20 h at 110°C in nitrogen flushed vacuum sealed ampules. Samples were run on a Beckman 6300 Amino Acid Analyzer. Stepwise regression was performed using SAS.

The procedure of Strange et al. with modifications was used to analyze samples by RP-HPLC on a Bechman HPLC System (420 Controller, Model 110A pumps, 340 Organizer, 164 Variable Wavelength Detector and 427 Integrator). Casein was dissolved in a 30% acetonitrile, .1% TFA, 4.5 M urea. 1  $\mu$ l  $\beta$ -mercaptoethanol/ml was also added to the sample solution. Bechman C3 and C8, 7.5 cm ultrapore columns were linked. A linear gradient from 30 to 50 percent acetonitrile containing .1% Trifluoroacetic acid was used to separate casein fractions.

The polyacrylamide gel electrophoresis procedure used was obtained from the lab of Dr. Juan Medrano. Samples were dissolved 1 mg / 100  $\mu$ l 6.6 M urea, 20  $\mu$ l .1% BPB and 20  $\mu$ l  $\beta$ -mercaptoethanol. Gels were scanned on an Instrument Specialties Company (ISCO) Gel Scanner Model 1312, (ISCO Model UA-5 Absorbance/Fluorescence Monitor) and Hewlett Packard 3390A Integrator.

Stepwise regression was used to predict whether a given variant was present in 0, 1 or both alleles based on normalized amino acid concentrations. An  $R^2$  of .83 was obtained using 12 amino acids for predicting the presence of  $\kappa$ -casein A or B as shown here. Amino acid analysis was not as good at predicting the  $\beta$ -casein variants present. The  $R^2$  values obtained reflect, in part, the use of casein genetic variant mixtures being used to predict whether no, one or both alleles contained a given casein variant. In addition, Van Eenennaam and Medrano dealing with  $\kappa$ -cas suggest that the two alleles of each casein may not be expressed equally. One allele may dominate which could also influence the ability of amino acid analysis to identify the presence and degree to which a variant is present.

The RP-HPLC procedure used may work to identify and quantify some of the casein genetic variants with further refinements. Previous amino acid analysis research has shown it can be used to predict the percentage of the various caseins present in a mixture. We did have limited success in predicting the  $\kappa$ -casein variant present and to a lesser extent the  $\beta$ -casein variants.



## Impact of Research:

The overall goal of our dairy products research program is to develop new dairy products by using new manufacturing processes. To do this, we must be able to measure the individual components in milk without separating them from milk. Methods available to determine casein and whey protein concentrations in milk require precipitation of casein followed by protein measurement of both precipitate and whey. Measurement of specific milk proteins, including genetic variants, calls for further separation by quantitative electrophoresis and protein determination of each fraction (Basch et al. 1985). These procedures are time consuming, expensive and susceptible to errors.

The goal of this project is to determine proportions of specific milk proteins (down to the level of specific genetic variants) or groups of proteins in milk and other dairy products from the information contained in a single amino acid analysis of a sample. Relative proportions of milk proteins found in traditional dairy products are subject to change as ultrafiltration and other new processes are used in their manufacture. To use these emerging manufacturing processes to produce entirely new products without the information that will be made available when this project is completed is nearly impossible. Many areas of research will also be facilitated by the results of this research. We will be able to follow milk protein composition through lactation periods of individual cows (or other species), correlate content of each of the milk proteins with coagulation properties during cheese making, make artificial infant formula that more closely matches mothers' milk, etc.

## References:

- Basch, J.J., F.W. Douglas, Jr., L.G. Procino, V.H. Holsinger, and H.M. Farrell, Jr. 1985. Quantitation of caseins and whey proteins of processed milks and whey protein concentrates, application of gel electrophoresis, and comparison with Harland-Ashworth procedure. *J. Dairy Sci.* 68:23-31.
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- Walsh, M.K. and R.J. Brown. 1988b. Separation and quantitation of casein proteins by reverse phase HPLC and amino acid analysis. Presented at the 83rd annual meeting of the American Dairy Science Assoc. Edmonton, Alberta.

**Abstracts:**

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- Walsh, M.K. and R.J. Brown. 1988b. Separation and quantitation of casein proteins by reverse phase HPLC and amino acid analysis. Presented at the 83rd annual meeting of the American Dairy Science Assoc. Edmonton, Alberta.
- Hollar, C.M., R.J. Brown, and J.F. Medrano. 1990. Quantification of casein genetic variants by RP-HPLC, PAGE, and amino acid analysis. J. Dairy Sci. 73 (Suppl. 1):80.

**Project Title:** Estimation of Individual Milk Proteins and Genetic Variants by Multicomponent Analysis of Amino Acid Profiles

**Personnel:** Rodney J. Brown, Dept. of Nutrition and Food Sciences, Utah State University

Carol H. Hollar, Dept. of Nutrition and Food Sciences, Utah State University

**Funding:** Western Dairy Foods Research Center

**Objectives:**

1. Determine concentrations of groups of proteins in milk such as caseins or whey protein using amino acid analysis.
2. Determine concentrations of specific milk proteins;  $\alpha$ s1,  $\alpha$ s2,  $\beta$ , and  $\kappa$ -caseins,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, bovine serum albumin using amino acid analysis.
3. Separate genetic variants of specific milk proteins, and use amino acid analysis to quantify individual variants in a protein mixture.
4. Use techniques developed in 1-3 analyze milk and other dairy products.
5. Determine mathematical procedures to obtain the most accurate and reproducible methods for estimating milk protein concentrations.

**Results:**

In the past year, whole casein was separated into  $\beta$ -casein,  $\kappa$ -casein,  $\alpha$ s1-casein and  $\alpha$ s2-casein fractions using cation-exchange fast protein liquid chromatography. The  $\gamma$ -caseins and several unidentified peaks were also separated. A urea-acetate buffer at pH 5 and a NaCl gradient from 0 to .26 M was used to separate the casein fractions. Several  $\gamma$ -caseins and unidentified fractions eluted first, followed by three  $\beta$ -casein peaks, several  $\gamma$ -casein and unidentified peaks,  $\kappa$ -casein,  $\alpha$ s1-casein and  $\alpha$ s2-casein. Some  $\gamma$ -caseins eluted with  $\beta$ -casein. The four major caseins, which accounted for over 90% of the whole casein fractions, were accounted for with this method and the calculated compositions correlated well with values obtained using anion-exchange fast protein liquid chromatography at pH 7.  $\beta$ -casein genetic variants A<sup>1</sup>, A<sup>2</sup> and B were separated using cation-exchange fast protein liquid chromatography.  $\beta$ -casein from a herd bulk casein sample eluted as a series of three peaks. Casein samples from individual cows containing known combinations of  $\beta$ -casein A<sup>1</sup>, A<sup>2</sup> and B were used to confirm that the three peaks were  $\beta$ -casein genetic variants. An acid-PAGE gel confirmed the identity of the peaks that eluted from the column.

**Impact of Research:**

The goal of this project is to determine proportions of specific milk proteins (down to the level of specific genetic variants) or groups of proteins in milk and other dairy products from the information contained in a single amino acid analysis of a sample. Relative proportions of milk proteins found in traditional dairy products are subject to change as ultrafiltration and other new processes are used in their manufacture. To use these

emerging manufacturing processes to produce entirely new products without the information that will be made available when this project is completed is nearly impossible. Many areas of research will also be facilitated by the results of this research. We will be able to follow milk protein composition through lactation periods of individual cows (or other species), correlate content of each of the milk proteins with coagulation properties during cheese making, make artificial infant formula that more closely matches mothers' milk, etc.

## **Publications:**

Hollar, C. H., R.J. Brown and J. F. Medrano. 1990. Quantification of casein genetic variants by RP-HPLC, PAGE, and amino acid analysis. 85<sup>th</sup> American Dairy Science Association Meeting J. Dairy Sci. 73:Supp. 1, 80.

Hollar, C. M., A. J. R. Law, D. G. Dalgleish, and R. J. Brown, 1991, Separation and quantification of major casein fractions using cation-exchange fast protein liquid chromatography. J. Dairy Sci. 74(8):2403-2409.

Hollar, C. M., A. J. R. Law, D. G. Dalgleish, and R. J. Brown, 1991, Separation of major casein fractions using cation-exchange fast protein liquid chromatography. 86<sup>th</sup> American Dairy Science Association Meeting J. Dairy Sci. 74:Supp. 1, 91.

Hollar, C. M., A. J. R. Law, D. G. Dalgleish, J. F. Medrano, and R. J. Brown, 1991, Separation of  $\beta$ -casein A1, A2 and B using cation-exchange fast protein liquid chromatography. 86<sup>th</sup> American Dairy Science Association Meeting J. Dairy Sci. 74:Supp. 1, 103.

Hollar, C. M., A. J. R. Law, D. G. Dalgleish, J. F. Medrano, and R. J. Brown, 1991, Separation and quantification of  $\beta$ -casein A<sup>1</sup>, A<sup>2</sup> and B using cation-exchange fast protein liquid chromatography. J. Dairy Sci. 74(9):0000-0000. In Press.

Hollar, C. M., A. J. R. Law, D. G. Dalgleish, and R. J. Brown, 1991, Separation and quantification of  $\kappa$ -casein variants using cation-exchange fast protein liquid chromatography. J. Dairy Sci. In Preparation.

Hollar, C. M., A. J. R. Law, D. G. Dalgleish, and R. J. Brown, 1991, Quantification of Casein Variants by Amino Acid Analysis. J. Dairy Sci. In Preparation.

**Project Title:** Evaluation of Iron-Protein Complexes in Iron-Fortified Dairy Products

**Personnel:** Arthur W. Mahoney, Professor, Department of Nutrition and Food Sciences, Utah State University

Mohan I. Reddy, Research Associate, Department of Nutrition and Food Sciences, Utah State University

**Funding Sources:** Western Dairy Foods Research Center  
Utah Agricultural Experiment Station

**Objectives:**

1. To determine the nature of the basic interaction of individual milk proteins, viz.,  $\alpha$ SI-casein, casein, K-casein, B-lactoglobulin,  $\alpha$ -lactalbumin and bovine serum albumen with ferrous sulfate ions, in simple buffer systems such as phosphate buffer at pH 6.6. Isolated proteins will be interacted with ferrous ions at pH 6.6 (pH of milk) and room temperature (25°C) to determine the kinetics of interaction, the rate of complex formation and the optimum iron/protein ratios for stable complex formation using equilibrium dialysis.
2. To study the effect of pH, temperature and ionic strength on the iron-protein complex formation for determining the optimum conditions for binding and stable iron-protein complex formation.
3. To determine the effect of iron binding on self-association of individual proteins and/or protein cross-binding. This will be of interest if whey proteins (B-Lg &  $\alpha$ -La) are cross-linked to caseins via chelation in terms of cheese yield.
4. To characterize the iron-protein complexes in Jennes-Koops buffer (32) (simulate milk salt buffer) using individual proteins as well as casein micelles and determine their stability. Also, the effect of iron binding on calcium (II) content of casein micelles will be studied.
5. To study the effect of iron binding to K-casein and casein micelles on the rennin hydrolysis of the phe-met bond of K-casein and subsequent coagulation of casein micelles.
6. To test the iron-protein complex formation in milk systems. Required amount of ferrous or ferric iron will be added to the milk system under controlled conditions and the complex formation will be allowed to occur. (This milk will be used for making yogurt and cheese.) The individual iron-protein complexes will be isolated from milk using ultracentrifugation and ion-exchange chromatography and characterized.
7. To determine the catalytic potency of iron-protein complexes on oxidative damage to model lipids and to lipids in milk, yogurt and cheese systems.

**Results:**

This project was funded 6 October 1988, but Dr. Mohan I. Reddy was not available until 1 July 1989. Therefore, research on this project is just being initiated.

### **Impact of Research:**

Iron deficiency continues to be a major nutritional problem because of inadequate iron intakes. Dairy products contain practically no iron. Furthermore, people who generally have high iron diets consume relatively small amounts of dairy products while people who consume larger amounts of dairy products have relatively low iron intakes. Iron fortification would increase the iron intakes of people who consume large amounts of dairy products, and it would allow people who are concerned with their iron nutriture to consume larger amounts of dairy products to achieve greater dietary calcium intakes. Thus, dairy products would be even more healthful in the diet if iron-fortified.

This research will provide basic information on the mechanisms of iron binding in dairy products, information essential to industrializing the technology of fortifying dairy products with iron. Work on this project was initiated 1 July 1989. We are now setting up the laboratory for dialysis experiments, developing dialysis methodologies, and purifying proteins to be used in the iron-binding studies, work necessary for completing the first year's objectives.

**Project Title:** Evaluation of Iron-Protein Complexes in Iron-Fortified Dairy Products

**Personnel:** Arthur W. Mahoney, Professor, Dept. of Nutrition and Food Sciences, Utah State University

Mohan I. Reddy, Research Associate, Nutrition and Food Sciences, Utah State University.

**Funding Sources:** Western Dairy Food Research Center  
Utah Agriculture Experiment Station

**Objectives:**

1. To determine the nature of the basic interaction of individual milk proteins, viz.,  $\alpha_{S1}$ -Casein,  $\beta$ -Casein, K-Casein,  $\beta$ -Lactoglobulin,  $\alpha$ -Lactalbumin and bovine serum albumin with ferrous/ferric iron in simple buffer systems such as Tris-HCl/phosphate buffer at pH 6.60. Isolated proteins will be interacted with ferrous/ferric iron at pH 6.60 (pH of milk) and room temperature ( $\sim 25^{\circ}\text{C}$ ) to determine the kinetics of interaction, the rate of complex formation and the optimum iron/protein ratios for complex formation using equilibrium dialysis/diafiltration technique.
2. To study the effect of pH, temperature and ionic strength on the iron-protein complex formation for determining the optimum conditions for binding and stable complex formation.
3. To determine the effect of iron binding on self-association of individual proteins and/or protein cross binding. This will be of interest if whey proteins ( $\beta$ -Lg &  $\alpha$ -La) are cross linked to caseins via chelation, in terms of cheese yield. This will be followed by gel filtration on H.P.L.C. and gel electrophoresis.
4. To characterize the iron-protein complexes in Jenness-Koops buffer (simulated milk salt buffer) using individual proteins as well as casein micelles and determine their stability. Also, the effect of iron binding on the calcium (II) content of casein micelles will be studied.
5. To study the effect of iron binding to K-Casein and casein micelles on the rennin hydrolysis of the phe-met bond of K-Casein and subsequent coagulation of casein micelles.
6. To test the iron-protein complex formation in milk systems. Required amount of ferric/ferrous iron will be added to the milk system under controlled conditions and the complex formation will be allowed to occur. (This milk will be used for making yoghurt and cheese.). The individual iron-protein complexes will be isolated from

milk using ultracentrifugation and ion-exchange chromatography (as described under the methods) and characterize them.

7. To determine the catalytic potency iron-protein complexes on oxidative damage to model lipids and to lipids in milk, yoghurt and cheese systems.

## Results:

Due to a number of problems unique to iron chemistry, the work on binding of iron to milk proteins did not progress as rapidly as expected. However, we have now developed the necessary methods and standardized conditions for testing iron binding to milk proteins in this laboratory. Therefore, we are poised to make excellent progress toward understanding iron chemistry of fortified cheese and other milk products.

Individual caseins, purified by mass-ion exchange chromatography on QAE-Sephadex, were used for binding studies. Initial iron-binding experiments using equilibrium dialysis indicated that this method was not suitable due to the protracted dialysis times (24 to 48h) common to this technique, which resulted in oxidation of iron salts leading to precipitation. Alternate methods such as gel filtration and diafiltration were tried and the latter method was found to be satisfactory owing to the shorter time duration (4 to 5h) required to complete a binding experiment. A schematic of the system used for diafiltration-binding studies is shown in Fig 1 and details of the diafiltration method are given in APPENDIX-I. Of the various ultrafiltration membranes screened for their suitability based on protein rejection and iron adsorption characteristics, PM-10 membrane was found to be ideal for these studies.

Using the diafiltration method, various iron salts were screened for their suitability in iron-protein binding experiments. Ferrous sulfate was not suitable due to rapid oxidation of the iron to the ferric state and precipitation. Ferric chloride underwent hydroxylation at pH 6.60 leading to the formation of insoluble  $\text{Fe}(\text{OH})_3$  polymers which did not pass through the ultrafiltration membrane. Ferric citrate also polymerized at pH 6.60 and was retained by the membrane. Ferric nitrilotriacetic acid,  $\text{Fe}(\text{III})\text{NTA}$ , prepared by adding solid ferric nitrate to a solution of sodium NTA (molar ratio of  $\text{Fe}:\text{NTA}$  of 1:2) was found suitable for iron binding studies.  $\text{Fe}(\text{III})\text{NTA}$  did not form polymers at pH 6.60 and freely passed through the PM-10 ultrafiltration membrane.

### Binding of Fe (III) to $\alpha_{S1}$ -Casein:

Studies on binding of  $\text{Fe}(\text{III})$  to  $\alpha_{S1}$ -Casein by diafiltration method using  $\text{Fe}(\text{III})\text{NTA}$  in PIPES (Piperazine-N, N'-bis[2-ethanesulfonic acid]) buffer containing 0.1M NaCl, pH 6.60 are in progress. A protein concentration of 5 mg/ml (0.22 mM) and an iron to protein molar ratios of 4.5 are being used in these studies. Binding curve showing the molar binding ratio ( $r$ , moles of  $\text{Fe}(\text{III})$  per mole of protein) vs free  $\text{Fe}(\text{III})$  concentration ( $A_f$ ) is given in Fig 2. Corresponding Scatchard plot is shown in Fig 3. The data indicate that  $\alpha_{S1}$ -Casein has two groups of non-identical binding sites, with  $n_1=3$  and  $n_2=11$ . Binding experiments at pH 5.50 resulted in precipitation of protein in diafiltration cell. Further studies on the effect of ionic strength, pH, and temperature are in progress.



## Impact of Research:

Iron deficiency continues to be a major nutritional problem because of inadequate iron intakes. Dairy products contain practically no iron. Furthermore, people who generally have high-iron diets consume relatively small amounts of dairy products while people who consume larger amounts of dairy products have relatively low iron intakes. Iron fortification would increase the iron intakes of people who consume large amounts of dairy products, and it would allow people who are concerned with their iron nutriture to consume larger amounts of dairy products to achieve greater dietary calcium intakes. Thus, dairy products would be even more healthful in the diet if iron-fortified.

This research will provide basic information on the mechanisms of iron binding in dairy products, information essential to industrializing the technology of fortifying dairy products with iron.

## APPENDIX I

**Diafiltration method.** Diafiltration system consists of a pressure source (nitrogen gas cylinder), a reservoir, a selector valve, a stirred cell assembly and a fraction collector. The standard cell assembly has a pressure inlet valve, a pressure relief valve, a membrane, and a filtrate outlet. The cell is placed on a magnetic stirrer. After proper assembly of the cell, the protein solution is poured into the cell, the lid is clamped on and the tubing from the selector valve is connected. The magnetic stirrer speed is adjusted to be vigorous enough to minimize concentration polarization of retained macromolecules.

In a typical experiment the protein solution (8.0 mL) is placed in the stirred cell and the Fe(III) NTA solution in the reservoir. By sliding the "gas" knob on the selector valve to allow the gas to first flow through the reservoir and then into the cell causes the solution in the cell and reservoir to become equally pressurized. By next sliding the "liquid" knob on the selector valve, the solution in the reservoir starts flowing from the reservoir into the cell. Some of the liquid in the cell permeates through the membrane and exits through the outlet tube. A vacuum is created and an equal volume of solution comes in from the reservoir into the stirred cell so that the volume of the protein solution in the cell remains constant. The permeate from the stirred cell assembly is collected in suitable volume fractions using a fraction collector and analyzed for iron content.

### Treatment of the binding data:

$$\text{Moles of iron in} = \text{Moles of iron out in the permeate} + \text{Moles of iron accumulated in cell} \quad (1)$$

$$\text{Moles of iron accumulated in the cell} = \text{Moles bound to the protein} + \text{Moles free iron in cell} \quad (2)$$

if	Sb	=	moles of iron bound to protein,
	A	=	moles of iron out in the permeate,
	V <sub>c</sub>	=	volume of protein solution in the stirred cell (liters),
	V <sub>p</sub>	=	volume of permeate out from stirred cell (liters),
	V <sub>r</sub>	=	volume of iron solution fed into the cell from the reservoir (liters),
	A <sub>r</sub>	=	concentration of iron in the reservoir (moles/liter),
	A <sub>p</sub>	=	concentration of iron in the permeate (moles/liter)

Then, at any given time

$$\text{Moles of iron in} = V_r \cdot A_r \quad (3)$$

Since  $V_r = V_p$ . Therefore

$$\text{Moles of iron in} = V_p \cdot A_r \quad (4)$$

Since the permeate concentration is the same as the cell concentration in a well-stirred system:

$$\text{Moles of free iron in the cell} = V_c \cdot A_p \quad (5)$$

Combining Eq. 1-5 :

$$V_p \cdot A_r = A + Sb + V_c \cdot A_p \quad (6)$$

$$Sb = V_p \cdot A_r - V_c \cdot A_p - A \quad (7)$$

The molar binding ratio ( $r$ ) could be determined as:

$$r = Sb/P \quad (8)$$

Where  $P$  is the concentration of the protein in moles.

The data was analyzed by the method of Scatchard, i.e., by plotting  $r$ , the number of moles of ligand (iron) bound per mole of protein, against  $r/[A_p]$ , where  $[A_p]$  is the free ligand concentration.

According to Scatchard,

$$r/[A_p] = nK - rK$$

where  $n$  and  $K$  are the total number of binding sites and the intrinsic binding constant, respectively. These values will be calculated from the intercept and slope of the plot, respectively.

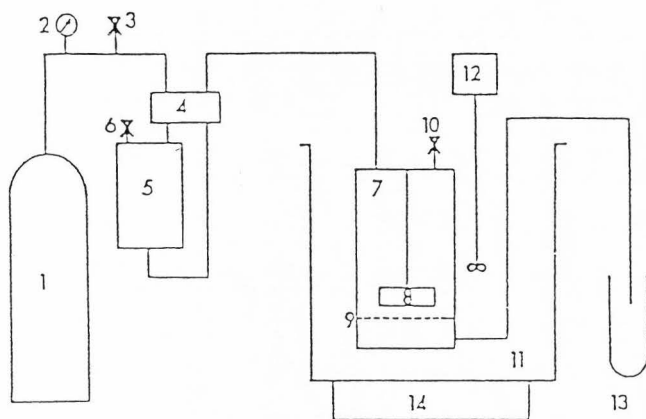


Fig 1. Schematic of Diafiltration System used for Binding Studies.

1. nitrogen cylinder; 2. pressure gauge; 3. pressure-relief valve (PRV); 4. three-way valve; 5. reservoir; 6. PRV; 7. diafiltration cell; 8. stirring bar; 9. membrane; 10. PRV; 11. water bath; 12. water bath agitation and temperature control; 13. permeate outlet and collection tube; 14. magnetic stirrer.

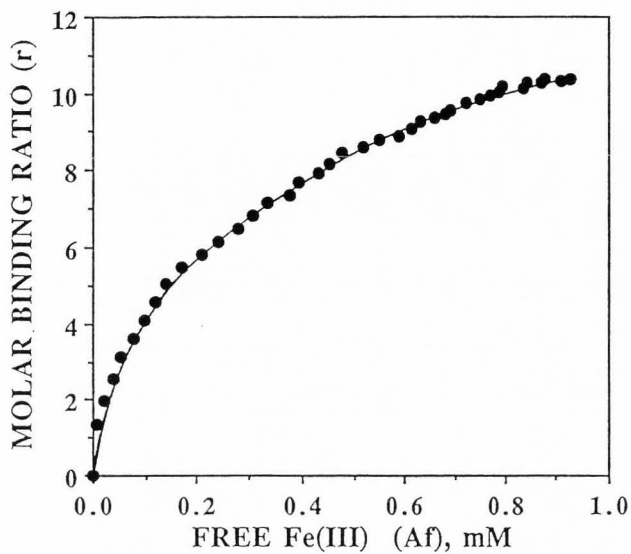


Fig 2. Binding Curve of Fe (III) to  $\alpha_s1$ -Casein at pH 6.60. Molar Ratio of Fe (III) to Protein is 4.5.

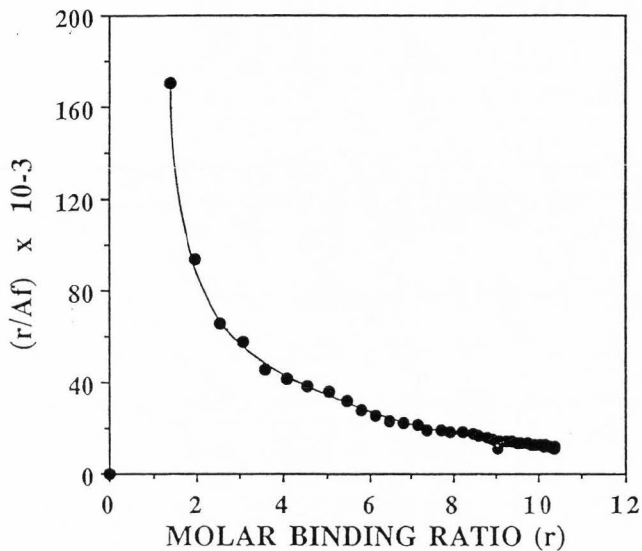


Fig 3. Scatchard Plot for the Binding of Fe (III) to  $\alpha_{S1}$ -Casein at pH 6.60. Molar Ratio of Fe (III) to Protein is 4.5.

## EVALUATION OF IRON-PROTEIN COMPLEXES IN IRON-FORTIFIED DAIRY PRODUCTS

**Personnel:** Arthur W. Mahoney, Ph.D., Professor  
Mohan I. Reddy, Ph.D.

### **Objectives:**

The objective of this study is to determine the chemistry of iron-protein complexes in milk as related to cheese making. We determined the chemical conditions under which maximal iron binding occurs (the conditions in which the iron is bound most tightly). This information is needed 1) for developing the best processing conditions which will give the highest quality iron-fortified dairy products and 2) for determining the best iron-protein complexes which will give the highest iron absorption from the fortified dairy products.

### **Results:**

**Diafiltration Binding Studies:** Binding of Fe(III) to individual caseins and whey protein fractions was studied. Molar binding ratio of Fe(III) to proteins increased as the free Fe(III) concentration increased. We found that  $\alpha_{s1}$ -,  $\beta$ -, and  $\kappa$ -caseins and BSA have two groups of non-identical binding sites with differing affinities for binding Fe(III). It appears that the first set of binding sites ( $n_1$ ) are preferentially filled, compared to the second set of binding sites ( $n_2$ ). No precipitation of proteins as a result of Fe(III) binding occurred even at saturating concentrations of Fe(III). The relative order of binding capacities for Fe(III) to casein and whey fractions was:  $\alpha_{s1}$ -casein >  $\beta$ -casein > BSA >  $\kappa$ -casein >  $\kappa$ -casein.

Further studies on the binding of Fe(III) to  $\alpha_{s1}$ -casein as a function of pH and ionic strength indicated that these parameters had no influence on the total number of binding sites, although binding affinity of Fe(III) to protein decreased with increase in pH from 5.60 to 7.80. Thus, from the practical point of view, the binding affinity of Fe(III) increases as the pH of milk is lowered by microbial action during cheese making. Some displacement of protein-bound Ca(II) by Fe(III) was observed for  $\alpha_{s1}$ -casein. Dephosphorylation of  $\alpha_{s1}$ -casein decreased the binding of Fe(III) to protein indicating that phosphoserine groups are involved in the binding.

**Visible-Difference Spectra of Fe(III)-Protein Complexes:** Difference absorption spectra of Fe(III)-protein complexes in the visible region (350 to 650 nm) was carried out to determine the possible groups involved in the binding of Fe(III) to different casein fractions and whey proteins. Negative absorption bands in the 420 nm region were observed for caseins indicating that phosphoserine groups and carboxyl groups of aspartic and glutamic acids are possible Fe(III) binding sites. The positive absorption band at 470 nm observed in caseins and whey proteins is typical of the absorption maximum of transferrin-iron complex and possibly due to a chelate site involving histidine and tyrosine residues. Thus, phosphoserines and carboxyl groups of aspartic and glutamic acids seem to play a major role in the binding of Fe(III) by caseins.

**Ultraviolet Difference and Fluorescence Spectra of Caseins and Whey Proteins:** Conformational changes in proteins, especially changes in the environment of aromatic side chains within proteins due to binding Fe(III) were monitored by following UV-difference spectra and fluorescence emission. The UV-difference spectrum of  $\alpha_{s1}$ -casein induced by Fe(III) had absorption bands at 210 nm indicating the possible involvement of tryptophan residues in charge-transfer type complex formation with Fe(III). Addition of Fe(III) to caseins and BSA caused a decrease in fluorescence intensity together with a red shift of the emission maximum. Iron addition to  $\beta$ -lactoglobulin caused a decrease in emission intensity without affecting emission the maximum. Studies on the

fluorescence emission spectra of  $\alpha_s1$ -casein as a function of Fe(III) concentration indicated progressive quenching together with a red shift of the emission maximum. This indicates that the binding of Fe(III) to proteins induced conformational changes resulting in the exposure of tyrosine and tryptophan residues to polar environment. However, not all the fluorophores (tryptophans) are uniformly quenched. All of the fluorophores are exposed to polar environment as a result of binding of Fe(III) to  $\alpha_s1$ -casein. Since conformational changes in general affect functional properties of proteins, it was of interest to see if binding of Fe(III) affects Ca(II) sensitivity of  $\alpha_s1$ -casein; addition of Fe(III) to  $\alpha_s1$ -casein increased the Ca(II) sensitivity of the protein.

**Effect of Fe(III) on the Renneting of Milk:** Since binding of Fe(III) induced conformational changes in milk proteins, it was of practical interest to see if addition of Fe(III) to milk during cheese making would affect renneting properties. Therefore, FeCl<sub>3</sub> was added to cold milk before pasteurization and to pasteurized milk (as in case of regular cheese making process) and chymosin hydrolysis and rennet clotting time were evaluated. We use approximately .20 mM FeCl<sub>3</sub> in making iron-fortified Cheddar cheese. Iron at this concentration had no effect on the chymosin hydrolysis of either milks. However, iron decreased the rennet clotting time of both whole and skim milk when added before pasteurization and increased the rennet clotting time when it was added to pasteurized skim milk. Iron barely affected the rennet clotting time of whole pasteurized milk. Thus, iron at the concentrations normally employed in making iron-fortified cheddar cheese, i.e., 0.2 mM, did not affect renneting properties of milk, and hence can be added to cheese milk. It was also observed that iron binding to casein micelles slightly decreased the calcium content of micelles, but it would not affect the calcium nutrition of cheese.

### Significance to the Dairy Industry

Iron fortification would increase the iron intakes of people who consume large amounts of dairy products, and it would allow people concerned with their nutrition to consume larger amounts of dairy products to achieve greater calcium intakes. Thus, dairy products would be even more healthful in the diet if iron-fortified. This research provides basic information on the mechanisms of iron binding in dairy products, information essential to industrializing the technology of fortifying dairy products with iron.

**Additional Findings:** UV-difference spectral and fluorescence spectral studies indicate that binding of Fe(III) to proteins induces conformational changes leading to the exposure of tryptophan and possibly tyrosine residues to polar environment. This is an interesting phenomenon because binding of Ca<sup>2+</sup> to  $\alpha_s1$ -, and  $\beta$ -caseins induces self aggregation leading to precipitation at high Ca<sup>2+</sup> concentrations whereas binding of Fe(III) to caseins does not result in precipitation. Further, binding of iron increased the Ca(II) sensitivity of  $\alpha_s1$ -casein. Change in conformation of caseins may have some implications in the functional properties of some cheeses such as mozzarella. Hence this issue needs to be addressed.

### Publications:

1. Reddy, M.I. and Mahoney, A.W. Effect of iron on chymosin hydrolysis and rennet clotting time of milk. Submitted to *J. Dairy Sci.*(1991).
2. Reddy, M.I. and Mahoney, A.W. Effect of iron on physico-chemical properties of casein micelles. In Preparation.
3. Reddy, M.I. and Mahoney, A.W. Preliminary studies on the binding of Fe(III) to bovine milk proteins. In Preparation.
4. Reddy, M.I. and Mahoney, A.W. Binding of Fe(III) to bovine  $\alpha_s1$ - casein. In Preparation.

5. Reddy, M.I. and Mahoney, A.W. A study of the interaction of Fe(III) with bovine  $\alpha_{S1}$ -casein as studied using ultraviolet and fluorescence spectroscopy. In Preparation.

Theses/Dissertations: Nil.

Abstracts:

1. Reddy, M.I. and Mahoney, A.W. Effect of iron on the renneting of milk. Accepted for presentation at '86th ADSA Annual Meeting' to be held at Utah State University, Logan, August 12-15, 1991.
2. Reddy, M.I. and Mahoney, A.W. Binding of Fe(III) to bovine  $\alpha_{S1}$ -casein. Accepted for presentation at '86th ADSA Annual Meeting' to be held at Utah State University, Logan, August 12-15, 1991.
3. Reddy, M.I. and Mahoney, A.W. A study of the interaction of Fe(III) with bovine  $\alpha_{S1}$ -casein using ultraviolet and fluorescence spectroscopy. Accepted for presentation at the 86th ADSA Annual Meeting' to be held at Utah State University, Logan, August 12-15, 1991.
4. Reddy, M.I. and Mahoney, A.W. Characterization of Fe(III)-milk protein complexes. Presented at The 23rd International Dairy Congress, Montreal, Canada, October 7-12, 1990.

Patents: Nil.



**Project Title:** Characterization of the Post-absorptive Behavior of  $\beta$ -lactoglobulin for Control of Spore and Microbial Adhesion to Dairy Product Processing and Packaging Surfaces.

**Personnel:** Joseph McGuire (PI), Assistant Professor, Agricultural Engineering and Food Science & Technology, Oregon State University

Kamal Al-malah, Graduate Research Assistant, Chemical Engineering, Oregon State University

Sidney Kirtley, Graduate Research Assistant, Food Science & Technology, Oregon State University

Viwat Krisdhasima, Graduate Research Assistant, Chemical Engineering, Oregon State University

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Kenneth R. Swartzel, Associate Professor, Food Science, North Carolina State University

**Funding Sources:** Western Dairy Foods Research Center  
Oregon Agricultural Experiment Station  
Oregon Agricultural Research Foundation  
AIChE Research Institute for Food Engineering  
National Science Foundation

### Objectives:

The project objectives, as originally stated are as follows.

1. to develop the mathematics required for a theoretically sound analysis of contact angle data for solid surface energy determination;
2. to use contact angle methods to evaluate the dispersive and polar components of solid surface energy for various materials;
3. use dynamic ellipsometry to continuously measure the changing thickness and refractive index of films adsorbed from an aqueous solution of B-lactoglobulin onto the characterized surfaces;

4. mathematically relate the conformational changes to time and solid surface energy;
5. combine B-lactoglobulin adsorption equilibrium data with the ellipsometric results to determine the unique protein conformation or range of conformations which bring about permanent adhesion of macromolecules, whole cells and spores; and
6. demonstrate the validity of predictive models.

## Results:

Project objectives 1 and 2 have been completed. We developed a methodology which requires only a simple contact angle goniometer equipped with an environmental chamber, and a series of aqueous ethanol solutions as diagnostic liquids. Several dairy contact materials were acquired and machined to a form suitable for study. The materials include many types of polymers, such as different polyethylenes, polypropylene, nylon, teflon, acetal, and others, along with stainless steel and glass.

The surface characterization methodology was recently published in detail; it can be briefly outlined as follows. Each surface under analysis is kept water-saturated to assure both equilibrium between the drop and the surface and the validity of the mathematics applied to the system. Diagnostic liquids are characterized with respect to their polar and dispersive components of surface tension, and the dispersive component of surface energy for each of the contact materials is evaluated. Using contact angle data recorded for each material, the polar component of the work of adhesion for each drop is evaluated and plotted against the polar component of the corresponding diagnostic liquid. A straight line always results; the slope of the line and its intercept are recorded, along with the work of adhesion between the solid surface and water, as evaluated using the straight line equation. These parameters, unique for each material, are mathematically combined with the previously determined dispersive component of solid surface energy and a composite surface energy is thereby constructed. Surface energetic parameters used to construct the composite surface energy provide sensitive indices of surface hydrophilicity and hydrophobicity, as well as material cleanliness.

This protocol for determination of contact surface properties will be used for the remaining phase of the project, which focuses on ellipsometry to monitor the influence of surface properties on the post-adsorptive behavior of B-lactoglobulin. Toward this end, cells which allow the continuous *in situ* observation of surface-induced protein conformational changes have been designed and constructed, and experiments are in progress. Also, funding has been received from other sources to study the surface equilibrium behavior of, B-lactoglobulin and the surface chemical nature of protein binding topics not covered under these Dairy Center project objectives, but essential to their successful completion.

This project is proceeding as scheduled and should be completed in December 1990 as originally planned. It represents a necessary and logical first step toward control of biofilm formation. In future work we intend to more directly address the protein-microbe interactions that occur during biofilm development, with continued concern for surface-protein interactions.

## Impact of Research:

It is the purpose of this research to quantify the post-adsorptive behavior of, B-lactoglobulin on several materials as a function of time and contact surface properties. An understanding of this

relationship should provide direction for the control of surface phenomena including biofilm development and associated bacterial colonization.

The danger associated with consuming foods contaminated by some microbial species is well-documented, and dairy foods have received wide attention as sources for such contamination in the food supply. Colonization and growth of microorganisms on dairy processing surfaces naturally presents a serious impediment to consistently providing wholesome, high quality milk products. In 1986 the FDA established the Dairy Safety Initiative Program to monitor commercial dairy products for *Listeria*. In its first year the surveillance project detected the organism in 2-3% of processed dairy products inspected, resulting in more than 40 product recalls. It is estimated that contaminated dairy products that could not be sold cost the dairy industry \$72 million in 1987.

Control of this problem seems best attained by focusing attention on the early events which occur at the interface and lead to biofilm formation. Support for this approach stems from the fact that microbial adhesion is suggested to be dependent upon the presence and conformational state of a pre-adsorbed, proteinaceous conditioning film on the contact surface. The conformational state of this pre-adsorbed film is dependent upon measurable properties of the original contact surface. Adsorbed protein which adopts a conformation approximating its native state is suspected to render a surface less susceptible to further film formation and microbial adhesion, whereas surface protein in a sufficiently denatured state invites further biofilm formation. A quantitative understanding of contact material surface properties and their relationship to the initial surface-protein and subsequent protein-microbe interactions associated with biofilm development will provide powerful direction for control of biofilm formation.

It seems clear that milk consumption cannot be enhanced if product quality and safety are not maintained. Moreover, product quality and safety are inseparable; quality can be assured only with active concern for safety of the food through the entire period associated with its production, processing, packaging and storage. Adverse publicity stemming from a listeriosis outbreak, for example, or even a single confirmed case, would likely generate a consumer reaction that is both negative and serious. Controlling the presence of undesired microorganisms in milk will undoubtedly serve to enhance its quality and safety. In the long run, an enhanced consumer confidence in dairy products will increase their utilization, serving to not only enhance sales of these products, but also to facilitate delivery of the highest quality product for a greater period of time. Indeed, delivery of the maximum product performance attainable as the sell-by-date is reached and surpassed is not possible without an understanding of the influence of contact surface properties on the interactions which bring about a deterioration in product quality. Finally, knowledge recently gained in this project with respect to interfacial chemistry should prove useful in selecting appropriate surface sterilizing systems, and should increase options available to dairy manufacturers by providing the information on which to base decisions for alternative packaging materials and sterilization strategies.

**Publications** (sponsored directly by the Dairy Center from 1 July 88 to 30 June 89):

#### Refereed Journal Articles

McGuire, J. and S.A. Kirtley, Surface Characterization for Prediction of Food Particle Behavior at Interfaces: Theoretical Considerations and Limitations. *Journal of Food Engineering*, 8(4): 273. 1988.

McGuire, J. and S.A. Kirtley, On Surface Characterization of Materials Targeted for Food Contact. *Journal of Food Science*, 54(1): 224. 1989.

Kirtley, S.A. and J. McGuire. On Differences in Surface Constitution of Dairy Product Contact Materials. *J. Dairy Sci.* 72: 1748. 1989.

McGuire, J. Temperature Influences on Food Contact Surface Energetics. In Engineering and Food, W.E.L. Spiess and H. Schubert (eds.), Elsevier Applied Science, New York and London. In press. 1989.

Lee, E. Temperature Effects on Surface Energetic Parameters Evaluated at Solid-Liquid Interfaces. M.S. Thesis, Oregon State University, Corvallis. 1989.

#### Proceedings and Symposia

McGuire, J. and S.A. Kirtley. Surface Characterization of Materials Targeted for Food Contact. Paper no. 43c, AIChE Summer National Meeting, Denver, CO. 1988.

McGuire, J. Temperature Influences on Food Contact Surface Energetics. In *Proceedings 5th International Congress on Engineering and Food*, Cologne, Federal Republic of Germany. Elsevier Applied Science, New York and London, in press. 1989.

Kirtley, S.A. and J. McGuire. Exploitation of Differences in Surface Constitution of Food Contact Materials. Paper no. 217, IFT Annual Meeting, Chicago, IL. 1989.

Krisdhasima, V., E. Lee, R.D. Sproull and J. McGuire. Temperature Influences on Food Contact Surface Properties. Paper no. 218, IFT Annual Meeting, Chicago, IL. 1989.

Krisdhasima, V., R.D. Sproull and J. McGuire. Application of Ellipsometry to the Study of Food Protein Behavior at Interfaces. Paper no. 702, IFT Annual Meeting, Chicago, IL. 1989.

**Project Title:** Characterization of the Post-adsorptive Behavior of  $\beta$ -lactoglobulin for Control of Spore and Microbial Adhesion to Dairy Product Processing and Packaging Surfaces

**Personnel:** Joseph McGuire (PI), Assistant Professor, Agricultural Engineering and Food Science & Technology, OSU

Viwat Krisdhasima, Graduate Research Assistant, Chemical Engineering, OSU

*other:*

Kamal Al-malah, Graduate Research Assistant, Chemical Engineering, OSU

Sidney A. Kirtley, Graduate Research Assistant, Food Science & Technology, OSU

Ja-Kael Luey, Graduate Research Assistant, Chemical Engineering, OSU

Robert D. Sproull, Assistant Professor, Chemical Engineering, OSU

Prasert Suttiaprasit, Graduate Research Assistant, Food Science & Technology, OSU

Kenneth R. Swartzel, Professor, Food Science, NCSU

Jianguo Yang, Graduate Research Assistant, Food Science & Technology, OSU

**Funding Sources:** Western Dairy Foods Research Center; Oregon Agricultural Experiment Station; Oregon Agricultural Research Foundation; AIChE Research Institute for Food Engineering; National Science Foundation

### **Objectives:**

The project objectives, as originally stated are as follows.

1. to develop the mathematics required for a theoretically sound analysis of contact angle data for solid surface energy determination;
2. to use contact angle methods to evaluate the dispersive and polar components of solid surface energy for various materials.

3. use dynamic ellipsometry to continuously measure the changing thickness and refractive index of films adsorbed from an aqueous solution of  $\beta$ -lactoglobulin onto the characterized surfaces;
4. mathematically relate the conformational changes to time and solid surface energy;
5. combine  $\beta$ -lactoglobulin adsorption equilibrium data with the ellipsometric results to determine the unique protein conformation or range of conformations which bring about permanent adhesion of macromolecules, whole cells and spores; and
6. demonstrate the validity of predictive models.

## Results:

Project objectives 1 and 2 were completed, and their results were reported last year. We have used these methods to evaluate surface energetic parameters related to surface hydrophilicity and hydrophobicity. The remaining phase of the project focuses on ellipsometry to monitor the influence of these surface properties on the post-adsorptive behavior of  $\beta$ -lactoglobulin. Toward this end, cuvettes that allow the continuous *in situ* observation of surface-induced protein conformational changes were designed and constructed; experiments are still in progress. In parallel, funding received from other sources to study the surface equilibrium behavior of  $\beta$ -lactoglobulin and the surface chemical nature of protein binding - topics essential to successful completion of the present project objectives (see Objective 5) - has been used to construct  $\beta$ -lactoglobulin adsorption isotherms. Surfaces have been modified to be hydrophilic by a series of oxidizing washings, and hydrophobic by surface methylation with dichlorodimethylsilane. We have successfully varied the degree of methylation on these surfaces to create a series of surfaces exhibiting varying hydrophobicities.  $\beta$ -lactoglobulin adsorption isotherms have been constructed on each of these surfaces as well; these will serve to assist interpretation of the equilibrium behavior observed on the metal, glass and polymer surfaces identified earlier. The influence of pH, ionic strength, and temperature on  $\beta$ -lactoglobulin adsorption equilibrium behavior has also been investigated. The most pertinent results of these investigations can be summarized as follows. In general, the amount of protein adsorbed at a surface was observed to increase with increasing surface hydrophobicity at all combinations of pH and ionic strength investigated. Moreover, these tests indicated that nonelectrostatic interactions between the surface and protein dominate the adsorption process at hydrophobic surfaces, while electrostatic interactions dominate the process at hydrophilic surfaces. As originally hypothesized, it appears that a surface-induced conformational change takes place upon adsorption, affecting the resultant adsorbed mass and film tenacity. The major unanswered question at this point is whether this change takes place in a single step immediately upon adsorption or whether some continuous change takes place after adsorption. The duration of the project is directed at resolving this question.

## Impact of Research:

It is the purpose of this research to quantify the post-adsorptive behavior of  $\beta$ -lactoglobulin on several materials as a function of time and contact surface properties. An understanding of this relationship should provide direction for the control of surface phenomena including bacterial biofilm development.

The danger associated with consuming foods contaminated by some microbial species is well-documented, and dairy foods have received wide attention as sources for such contamination in the food supply. Colonization and growth of microorganisms on dairy processing surfaces naturally presents a serious impediment to consistently providing wholesome, high quality milk products. Control of this problem seems best attained by focusing attention on the early events that occur at the interface and lead to biofilm formation. Support for this approach stems from the fact that microbial adhesion is suggested to be dependent upon the presence and conformational state of a pre-adsorbed, proteinaceous conditioning film on the contact surface. The conformational state of this pre-adsorbed film is dependent upon measurable properties of the original contact surface. Adsorbed protein that adopts a conformation approximating its native state is suspected to render a surface less susceptible to further film formation and microbial adhesion, whereas surface protein in a sufficiently denatured state invites further biofilm formation. A quantitative understanding of contact material surface properties and their relationship to the initial surface-protein and subsequent protein-microbe interactions associated with biofilm development will provide powerful direction for control of biofilm formation.

It seems clear that milk consumption cannot be enhanced if product quality and safety are not maintained. Moreover, product quality and safety are inseparable; quality can be assured only with active concern for safety of the food through the entire period associated with its production, processing, packaging and storage. Adverse publicity stemming from a listeriosis outbreak, for example - or even a single confirmed case - would likely generate a consumer reaction that is both negative and serious. Controlling the presence of undesired microorganisms in milk will undoubtedly serve to enhance its quality and safety. In the long run, an enhanced consumer confidence in dairy products will increase their utilization. Finally, knowledge recently gained in this project with respect to interfacial chemistry should prove useful in selecting appropriate surface sterilizing systems, and should increase options available to dairy manufacturers by providing the information on which to base decisions for alternative packaging materials and sterilization strategies.

## Publications:

McGuire, J. and S.A. Kirtley. Surface Characterization for Prediction of Food Particle Behavior at Interfaces: Theoretical Considerations and Limitations. *Journal of Food Engineering*. 8: 273-286. 1988.

- McGuire, J. A Predictive Model for Food Particle Interactions with Contact Surfaces. *Journal of Food Science*. 54: 22-24,29. 1989.
- McGuire, J. and S.A. Kirtley. On Surface Characterization of Materials Targeted for Food Contact. *Journal of Food Science*. 54: 224-226. 1989.
- McGuire, J. Elucidation of the Surface Influence on Irreversible Protein Adsorption. *Biofouling*. 1: 263-267. 1989.
- McGuire, J. and K.R. Swartzel. The Influence of Solid Surface Energetics on Macromolecular Adsorption from Milk. *Journal of Food Processing and Preservation*. 13: 145-160. 1989.
- Kirtley, S.A. and J. McGuire. On Differences in Surface Constitution of Dairy Product Contact Materials. *Journal of Dairy Science*. 72:1748-1753. 1989.
- Lee, E. *Temperature Effects on Surface Energetic Parameters Evaluated at Solid-Liquid Interfaces*. M.S. Thesis, Oregon State University. 104 pages. 1989.
- Luey, J. *The Effect of pH and ionic Strength on the Adsorption of  $\beta$ -lactoglobulin onto Well-Characterized Silicon*. M.S. Thesis, Oregon State University. 53 pages. 1990.
- McGuire, J. Temperature Influences on Food Contact Surface Energetics. In *Engineering and Food*, W.E.L. Spiess and H. Schubert (eds.), Elsevier Applied Science, New York and London. **In press**.
- McGuire, J. and R.D. Sproull. Temperature Effects on Food Contact Surface Properties. *Journal of Food Science*. In press.
- McGuire, J. On Evaluation of the Polar Contribution to Contact Material Surface Energy. *Journal of Food Engineering*. In press.
- McGuire, J., K. Al-malah, F.W. Bodyfelt and M.J. Gamroth. Application of Ellipsometry to Evaluation of Cleaning Effectiveness. *Journal of Food Science*. **In press**.
- McGuire, J., E. Lee and R.D. Sproull. Temperature Influences on Surface Energetic Parameters Evaluated at Solid-Liquid Interfaces. *Surface and Interface Analysis*. **In press**.

## Abstracts:

- McGuire, J. A Predictive Model for Food Particle Interactions with Contact Surfaces. Paper no. 80, IFT Annual Meeting, New Orleans, LA. 1988.



- McGuire, J. and S.A. Kirtley. Surface Characterization of Materials Targeted for Food Contact. Paper no. 43c, AIChE Summer National Meeting, Denver, CO. 1988.
- Krisdhasima, V., P. Suttiprasit, K. Al-malah, J. McGuire and R.D. Sproull. Post-adsorptive Behavior of  $\beta$ -lactoglobulin. 78th Annual ODI Conference, Eugene, OR. 1989.
- McGuire, J. Temperature Influences on Food Contact Surface Energetics. Paper no. 22.20, 5th International Congress on Engineering and Food, Cologne, Federal Republic of Germany. 1989.
- Kirtley, S.A. and J. McGuire. Exploitation of Differences in Surface Constitution of Food Contact Materials. Paper no. 217, IFT Annual Meeting, Chicago, IL. 1989.
- Krisdhasima, V., E. Lee, R.D. Sproull and J. McGuire. Temperature Influences on Food Contact Surface Properties. Paper no. 218, IFT Annual Meeting, Chicago, IL. 1989.
- Krisdhasima, V., R.D. Sproull and J. McGuire. Application of Ellipsometry to the Study of Food Protein Behavior at Interfaces. Paper no. 702, IFT Annual Meeting, Chicago, IL. 1989.
- Krisdhasima, V., P. Suttiprasit, J. McGuire and R.D. Sproull. Post-adsorptive Behavior of  $\beta$ -lactoglobulin. 79th Annual ODI Conference, Eugene, OR. 1990.
- Suttiprasit, P. and J. McGuire. Relative Interfacial Behavior of Milk Proteins at Hydrophobic and Hydrophilic Surfaces. Paper no. 37, IFT Annual Meeting, Anaheim, CA. 1990.
- Al-malah, K. and J. McGuire. Application of Ellipsometry to the Characterization of Milk Soils on Fouled and Cleaned Surfaces. Paper no. 39, IFT Annual Meeting, Anaheim, CA. 1990.
- Yang, J., J. McGuire and E.R. Kolbe. Use of the Equilibrium Contact Angle as an Index of Contact Surface Cleanliness. Paper no. 154, IFT Annual Meeting, Anaheim, CA. 1990.
- Al-malah, K., J. Luey, J. McGuire and R.D. Sproull.  $\beta$ -lactoglobulin Adsorption Equilibrium at Solid Surfaces, to be presented at the AIChE Summer National Meeting, San Diego, CA. 1990.
- Krisdhasima, V., J. McGuire and R.D. Sproull. Surface-Induced Conformational Changes Experienced by  $\beta$ -lactoglobulin at Hydrophilic and Hydrophobic Surfaces, to be presented at the AIChE Summer National Meeting, San Diego, CA. 1990.

Wang, D.Q., E.R. Kolbe, J. McGuire and R.D. Sproull. Kinetic Analysis of Freeze Denaturation of Pacific Red Rockfish Myofibrillar Protein Using DSC, to be presented at the AIChE Summer National Meeting, San Diego, CA. 1990.

**Project Title** Characterization of the post-adsorptive behavior of  $\beta$ -lactoglobulin for control of spore and microbial adhesion to dairy product processing and packaging surfaces

**Personnel:** Joseph McGuire, Bioresource Engineering and Food Science & Technology, Oregon State University

Wiwat Krisdhasima, Chemical Engineering and Food Science & Technology, Oregon State University

**Funding:** Western Dairy Foods Research Center, AIChE Research Institute for Food Engineering, National Science Foundation, Public Health Service Institutional Grant (OSU), Oregon Agricultural Experiment Station

### Objectives:

The project objectives for fiscal year 1991 were to use dynamic ellipsometry to continuously measure the changing thickness and refractive index of  $\beta$ -lactoglobulin films adsorbed from aqueous solution, and to relate changes in surface conformational state to time and solid surface properties.

### Results:

We have used ellipsometry to monitor the influence of contact surface hydrophobicity on the post-adsorptive behavior of  $\beta$ -lactoglobulin ( $\beta$ -lg). Cuvettes that allow the continuous *in situ* observation of surface-induced protein conformational changes were designed and constructed for that purpose. Silicon surfaces have been modified to be hydrophilic by a series of oxidizing washings, and hydrophobic by surface methylation with dichlorodimethylsilane. We have successfully varied the degree of methylation on these surfaces to create a series of surfaces exhibiting varying hydrophobicities.  $\beta$ -lg adsorption isotherms have been constructed for each of these surfaces as well.

In parallel with the Center-sponsored work, funding received from other sources has been used to construct  $\beta$ -lg adsorption isotherms on acrylic, glass, polycarbonate, polyester and #304 stainless steel surfaces (Al-Malah *et al.*, 1991). The influence of pH and ionic strength on  $\beta$ -lg adsorption equilibrium has also been investigated (Luey *et al.*, 1991).

As originally hypothesized, it appears that a surface-induced conformational change takes place upon  $\beta$ -lg adsorption. We formulated a simple model for protein adsorption, accounting for an initial reversible step followed by a conformational change generating an irreversibly adsorbed species. We derived an expression for adsorbed mass as a function of time, protein concentration, and reaction rate constants, then used nonlinear regression to fit *in situ* adsorption kinetic data to our derived expression. The model described adsorption well in all cases with statistical analysis indicating that all parameters are significant. As expected, plateau values of adsorbed mass were observed to increase with increasing surface hydrophobicity. This is consistent with the observation that protein adsorption is an entropically driven process, and in agreement with our own equilibrium studies.

Expressions describing the kinetic rate constants that define protein arrival and unfolding support the hypothesis that rates of arrival and unfolding increase with increasing solid surface hydrophobicity, while the tendency of adsorbed protein to desorb decreases.

## Impact of Research:

It was the purpose of this research to quantify the post-adsorptive behavior of  $\beta$ -lg on several materials as a function of time and contact surface properties. This purpose was fulfilled. Interpretation of adsorption kinetics with reference to a molecular model was shown to yield useful relationships among the rate constants that describe interfacial behavior. We are now prepared to seriously address the issue of what protein surface activity really has to do with bacterial adhesion. Based on the type of experimentation described in a new project proposal to the Center (M.A. Daeschel and J. McGuire, co-PIs), knowing the protein content of a fluid product, we should be able to describe surface and protein chemical influences on bacterial adhesion in a truly quantitative manner.

## Publications:

- McGuire, J. and R.D. Sproull. Temperature effects on food contact surface properties. *J. Food Sci.* **55**: 1199-1200. 1990.
- McGuire, J. On evaluation of the polar contribution to contact material surface energy. *J. Food Engng.* **12**: 239-247. 1990.
- McGuire, J., E. Lee and R.D. Sproull. Temperature influences on surface energetic parameters evaluated at solid-liquid interfaces. *Surf. Interface Anal.* **15**: 603-608. 1990.
- Luey, J., J. McGuire and R.D. Sproull. The effect of pH and NaCl concentration on adsorption of  $\beta$ -lactoglobulin at hydrophilic and hydrophobic silicon surfaces. *J. Colloid Interface Sci.* **143**: 489-500. 1991.
- Al-malah, K., J. McGuire, V. Krisdhasima, P. Suttiaprasit and R. Sproull. Ellipsometric evaluation of  $\beta$ -lactoglobulin adsorption onto low- and high-energy materials. *Biotechnol. Progr.* (submitted).

**Project Title:** Method for Identifying Batch of Origin in Semi-Continuous Cheese Making Processes

**Personnel:** L. V. Ogden, Associate Professor, Food Science and Nutrition, Brigham Young University

Mike Dunn, Graduate Assistant, Food Science and Nutrition, Brigham Young University.

**Funding Sources:** Western Dairy Foods Research Center, non-dairy board monies  
Brigham Young University College of Biology and Agriculture  
Department of Food Science and Nutrition.

**Objectives:**

This study will lay the ground work for on-line colorimetric batch identification by identification of markers that are maximally detectable to a Hunter Labscan II Colorimeter in either the L,a, or b dimension while being minimally detectable to consumers. It will establish how much marker will be necessary in alternate batches to facilitate confident identification of the "seam" between batches. Variable levels of annatto, and addition of beta carotene, turmeric, and canthaxanthan will be investigated as possible markers. Each marker will be evaluated and compared with respect to cost, ease of incorporation, level of detectability, test reliability, and possible side effects.

**Results:**

Beta carotene, canthaxanthan (another carotenoid), turmeric, and annatto (at increased levels) were the colors selected for use as batch markers in the experimental system.

The experimental system consisted of three four-gallon vats contained in a heated water bath. Thirty pounds of milk was added to each vat. After inoculation with starter, all three vats were colored with 1.2 ml. of annatto. Two of the vats received additional coloration with a prepared marker solution, one at a lower level, and one at a higher level. Cheddar cheese manufacture was continued following a stirred curd make procedure. The result was three batches of cheese representing three different marker concentrations, one of which was a 0 level control (1.2 ml. annatto only).

Immediately after removing the cheese from the press, four readings were taken on each of the three batches using a Hunter Labscan II colorimeter. Hunter L, a, and b values as well as spectral data were collected. By repeating this entire process twice (a total of three times) for each of the four markers. A standard curve was plotted for each marker using either the L a, b values or the spectral data. Ninety-five percent confidence limits were established for each curve. The minimum marker concentration needed to obtain a significant colorimetric difference was determined by setting the upper or lower limit of the control (depending on the slope of the line) and solving for the actual marker value. The back to back confidence limits combine to keep the confidence at 95%.

By following the preceding approach, minimally detectable marker levels were determined for each of the four colors. The next step will be to carry our sensory panels on batches of cheese made at these levels to determine what effect marker addition will have on the overall sensory properties of the cheese.

**Impact of Research:**

Inability to accurately identify the batch of origin of specific units of cheese for recall or downgrading is a problem in plants using cheddaring and/or block forming towers in cheese manufacturing. One plant manager estimated the unnecessary value reduction on his cheese to be \$250,000 per year assuming that 6.5 million pounds of cheese was unnecessarily downgraded and an average reduction in value of \$.04 per pound. Ability to identify batch of origin would prevent that loss.

**Project Title:**                    **Method for Identifying Batch of Origin in Semi-Continuous Cheese Processes**

**Personnel:**                    Lynn V. Ogden, Principal Investigator, Dept. of Food Science and Nutrition, Brigham Young University

**Funding Sources:**        Western Dairy Foods Research Center

### **Summary:**

The feasibility of using minimal color variations in alternate batches and on-line colorimetry to detect the seams between batches was investigated as a means of determining the batch or origin in semi-continuous cheese processes. Four vegetable colors were investigated in small laboratory batches using the Hunter Labscan 2. Amounts of color addition necessary for a detectable colorimetric difference and resulting relative visual difference were determined. A plant trial was conducted varying the amount of annatto in alternate batches.

### **Results:**

In lab trials, turmeric was the most sensitive marker and canthaxanthin the least sensitive with annatto and beta carotene in the middle. A 19% change in annatto usage was found to be necessary to detect the change colorimetrically using the Hunter b value. Nine and sixteen percent changes in annatto were tried in a plant trial using Yellow Index, Hunter b values, and the Hunter Qual probe as colorimetric measures. Colorimetric and visual sensitivity to these variations is being analysed.

### **Publications:**

Dunn, M.L. 1989. Method of Identifying the Batch of Origin in Semi-Continuous Cheesemaking Processes. Masters Thesis, Department of Food Science and Nutrition, Brigham Young University.

Manuscript ready for submission to Journal of Dairy Science.

### **Significance to the Dairy Industry:**

Accurate determination of batch of origin is needed in semi-continuous cheese processes to confine downgrading to only that cheese produced in defective batches. Currently the equivalent of three batches are downgraded to assure that all one defective batch is included.

**Project Title:** Optimization of the Sensory Characteristics of Flavored Yogurt

**Personnel:** Mina R. McDaniel, Associate Professor, Food Science and Technology, Oregon State University  
Floyd W. Bodyfelt, Professor, Food Science and Technology, Oregon State University  
Nancy J. Micheals, Research Assistant, Food Science and Technology, Oregon State University  
Steven J. Harper, Graduate Student, Food Science and Technology, Oregon State University  
Debbie L. Barnes, Graduate Student, Food Science and Technology, Oregon State University

**Funding Sources:** Western Dairy Foods Research Center

**Research Objectives:**

**A. Trained Panel Descriptive Analysis**

1. To train a panel for and to rate sweetness, sourness and flavor attributes of a variety of Pacific Northwest commercial yogurts of different flavors using reference standards of sucrose, lactic acid, and flavoring systems.
2. To determine the sweetness/sourness ratios for the commercial yogurts.
3. To develop terminology to describe the body and texture of the samples, and complete a texture profile analysis of the samples to measure graininess, chalkiness, firmness, viscosity, gel character, etc.
4. To statistically correlate taste and texture descriptor ratings across all commercial products tested.

**B. Analytical and Physical Testing**

1. To analyze all samples for pH, titratable acidity, sugar, acid, and acetaldehyde using appropriate methods.
2. To correlate pH, titratable acidity and sugar analysis data with sensory descriptors.
3. To observe relationships between the sugar/acid ratios and the sweetness/sourness ratios.
4. To analyze for viscosity and other appropriate textural attributes.

**C. Consumer Panel**

1. To develop a ballot for consumer evaluation of the commercial yogurt samples.



2. To run replicate 50 member panels for evaluation of each set of samples for ascertaining if preferences exist among the samples, and to determine directional information for product improvement.
3. To evaluate the effective demographic factors such as age, sex, yogurt consumption patterns, etc.
4. To correlate consumer data with trained panel, analytical and physical measurements,
5. To study the acceptance of sundae-style yogurts with a separate consumer test.

#### D. Formula Optimization

1. To determine ranges of important flavor and textural qualities, and to prepare yogurt formulas incorporating these ranges.
2. To optimize the product formula using appropriate statistical techniques in a series of large consumer style sensory tests.

#### Results:

A pilot study was performed to gain information about and to pretest the methodology for conducting the above research. This study began with a preliminary interview survey to determine the effectiveness of the survey format, and to gain preliminary demographic information.

A trained descriptive panel and consumers evaluated five different commercial brands of strawberry yogurt (3 sundae style, and 2 pre-stirred). From the trained panel, significant differences were found for all attributes tested with the exception of acetaldehyde. Especially large differences were found for color intensity, lumpiness and viscosity by appearance, amount of fruit pieces, overall flavor intensity, lumpiness and viscosity by mouthfeel, artificial strawberry flavor, sweetness and sourness. In comparison with the other yogurts:

Yogurt 1 was characterized by high color intensity, overall flavor intensity, and artificial strawberry flavor while being low in lumpiness by appearance and mouthfeel, and amount of fruit pieces;

Yogurt 2 showed a high level of size of fruit pieces, sweetness, and artificial strawberry flavor while being low in color intensity, viscosity and lumpiness by appearance and mouthfeel, amount of fruit pieces and sourness;

Yogurt 3 had a high level of chalkiness, fresh strawberry flavor and amount of fruit pieces, and a low level of sourness and artificial strawberry flavor;

Yogurt 4 was low in color intensity, but high in lumpiness by appearance, astringency, viscosity by appearance and mouthfeel, chalkiness, sourness, and fresh strawberry flavor;

Yogurt 5 was characterized by high levels of lumpiness by mouthfeel, amount of fruit pieces, sourness and fresh strawberry flavor, and low levels of overall flavor intensity, jammy and artificial strawberry flavor, size of fruit pieces and sweetness.

Overall, yogurts 1 and 2, as well as yogurts 4 and 5, were similar in many attributes. Principal component analysis or Discriminant analysis could be used to verify similarity of these yogurts.

The consumer test utilized six attributes, three analyzed on a 9-pt. hedonic scale and three on a just right scale for the yogurts. Yogurt 1 was rated highest in liking by consumers for overall impression, appearance and overall texture, and was rated near just right for sweetness, sourness and strawberry flavor. Yogurt 2 was similar to yogurt 1 except for having a slightly lower rating for sourness and strawberry flavor. Yogurt 3 also had a high degree of liking for appearance compared with 1 and 2, but was lower in overall impression, texture, and sweetness, and was just right for the sourness level. Yogurt 4 was rated like slightly for appearance, overall impression, and texture, and showed lower sweetness and higher sourness ratings. Yogurt 5 was lowest in all attributes except appearance, and had the highest degree of sourness. Looking at the descriptive and consumer data together allows us to characterize each yogurt.

This pilot study was a good starting point for examining the techniques and methods to be used for the actual descriptive and consumer studies in combination with simultaneous analytical and physical tests. Further research will concentrate on the objectives stated above using refined survey and consumer ballot techniques, and a more highly trained panel. Other flavors and more brands will be studied using sensory and analytical methods to determine optimal formulas for the sweetness/sourness ratios.

### **Impact of Research:**

If accepted and utilized by the yogurt manufacturers this research will provide information regarding acceptance of the product by the consumers. An optimized formula could increase sales to both consumers and non-consumers of yogurt, consumption of fluid milk, and competition with national brands. If the demographic results indicate distinct populations of yogurt consumers, a better understanding of consumers desires will be noted, and advertising could be targeted to these groups to help increase consumption. In the United States, currently, yogurt consumption is low per capita compared to the European countries such as the Netherlands indicating the potential for increased acceptance and sales.

**Project Title:** Optimization of the Sensory Qualities of Flavored Yogurt

**Personnel:** Mina R. McDaniel, Principal Investigator, Associate Professor, Department of Food Science and Technology, Oregon State University

Floyd W. Bodyfelt, Co-Investigator, Professor, Department of Food Science and Technology, Oregon State University

Nancy Michaels, Research Assistant, Department of Food Science and Technology, Oregon State University

Debbie L. Barnes, Graduate Student, M.S., Department of Food Science and Technology, Oregon State University

Steven J. Harper, Graduate Student, Ph.D., Department of Food Science and Technology, Oregon State University

**Funding Sources:** Western Dairy Foods Research Center

**Objectives:**

A. Trained Panel Descriptive Analysis

1. To train a panel for and to rate the sweetness, sourness, flavor intensity and aroma intensity of Pacific Northwest commercial yogurts of different flavors (strawberry, raspberry, lemon and plain) using a 1 6-pt. intensity.
2. To determine the sweetness/sourness ratios of the commercial yogurts .
3. To develop terminology to describe the body and texture of the yogurt samples and to then complete a texture profile analysis of the commercial samples to measure graininess, chalkiness, firmness, viscosity, gel character, etc.
4. To correlate taste and texture descriptor ratings across all commercial products tested.
5. To complete analysis of variance on all data collected.

## B. Analytical and Physical Testing

1. To analyze all samples for pH and titratable acidity by standard methods .
2. To analyze for sugars content using HPLC.
3. To correlate pH, titratable acidity, and sugar analysis data with sensory descriptors.
4. To observe relationships between the sugar/acid ratios and the sweetness/sourness ratios.
5. To analyze for viscosity and other appropriate textural attributes .

## C. Consumer Panel

1. To develop a ballot for consumer evaluation of the commercial yogurt samples.
2. To ascertain if differences in preference exist among the samples, and to determine directional information for product improvement .
3. To complete statistical analysis to evaluate the effective demographic factors such as age, sex, and yogurt consumption patterns .
4. To correlate consumer acceptance data with trained panel, analytical and physical measurements.

## D. Formula Optimization

1. To determine, using results from the above studies, ranges of all important flavor qualities, and to prepare yogurt formulas incorporating these ranges.
2. To optimize the product formula using Response Surface Methodology test design in a series of large consumer style sensory tests.

## Results:

As the objectives above were written in the form of a two year project, the following results are for the work completed by the time of this annual report. At the end of this project year, much of the work on aroma and flavor has been completed, but texture work and optimization work remains. At this point we have enough results to write and submit four abstracts of the work accomplished to date. One paper will be presented at the Institute of Food Technologists in Anaheim in June, and three will be presented the following week at the American Dairy Science Meeting in North Carolina. To briefly summarize the findings, the following statements apply:

1. A large range in overall acceptability (rated on a 9-pt Hedonic Scale ranging from 1 = dislike extremely to 9 = like extremely) was found for each set of yogurts tested. Overall liking ranged from 5.69 to 7.89 for strawberry yogurts, 4.97 to 7.57 for lemon yogurts, 4.92 to 7.63 for raspberry yogurts, and 5.38 to 7.50 for plain yogurts. Only consumers of these products were tested.
2. Large differences were found between all brands of strawberry yogurts for both descriptive panel attributes and consumer hedonic ratings. The yogurts have very different flavor profiles with predominant flavor attributes being different between samples.
3. Consumer overall impression ratings were determined by how well they liked the strawberry flavor, sweetness and sourness balance of the sample. Two groups of opposing attributes appeared to play predominant roles. Sweetness, jammy strawberry flavor and cooked milk attributes were positively correlated with acceptability while plain yogurt, acetaldehyde, sourness and astringency were negatively correlated.
4. Bitterness was responded to negatively by consumers and reduced acceptability.
5. Only one lemon yogurt sample was found to significantly less acceptable from consumer hedonic ratings. This sample rated lowest in lemon flavor, was moderately too sour, and was moderately not sweet enough.
6. Significant correlations were found in consumer ratings between overall liking, lemon flavor, sweetness and sourness with highest correlations to lemon flavor and sweetness. Consumers seem to be most concerned with flavor and sweetness
7. Significant sample differences resulted for all descriptive panel attributes. Two opposing groups of attributes seemed to affect descriptive results. Sourness, plain yogurt, acetaldehyde, and astringency were correlated with each other as were sweetness, overall lemon, lemon juice, and artificial lemon. The sample rated the lowest in overall liking rated high in attributes from the first group while the sample rated the highest in overall liking rated high in attributes from the second group.
8. There was a wide range of consumer hedonic ratings for overall liking in plain yogurt. Overall liking was positively correlated with appearance, texture, sweetness and sourness liking. Sweetness and sourness liking were most highly correlated ( $p < .0001$ ).
9. "Just Right" scale analysis for plain yogurt revealed that samples with highest overall liking ratings had sweetness and sourness ratings closest to "just right".

10. Attention to appearance, texture and the sweetness/sourness balance in plain yogurt appears to be important for consumer acceptability.

### **Impact of Research:**

This study demonstrated that there were very large differences in acceptability among the commercial samples tested for all yogurt flavors evaluated. These findings support the need for optimization work, and the need for improvement of the sensory qualities of commercial yogurts. Optimization work, if continued, could document changes that would improve yogurt quality and increase consumer acceptability, ultimately leading to increased yogurt sales.

### **Abstracts:**

Barnes, D. L., Harper, S. J. and McDaniel, M. R. Correlation of descriptive and consumer panel flavor ratings for commercial pre-stirred strawberry yogurts. Institute of Food Technologists, Anaheim, CA, June, 1990.

Harper, S. J., Barnes, D. L., McDaniel, M. R. Correlation of descriptive and consumer panel flavor ratings for commercial pre-stirred lemon yogurts. American Dairy Science Association, Raleigh, NC, June, 1990.

Harper, S. J., Barnes, D. L., McDaniel, M. R. Sensory attribute ratings of commercial plain yogurts by consumer and descriptive panels. American Dairy Science Association, Raleigh, NC, June, 1990.

Barnes, D. L., Harper, S. J., McDaniel, M. R. The effect of commercial yogurt sweet/sour ratios on consumer acceptance. American Dairy Science Association, Raleigh, NC, June, 1990.

- Project Title:** Acid whey utilization: Functional properties of a food grade stabilizer produced by *Lactobacillus plantarum* from acid whey
- Personnel:** J. Antonio Torres, Principal Investigator, Dept. of Food Science and Technology, Oregon State University
- Mark A. Daeschel, Co-Investigator, Dept. of Food Science and Technology, Oregon State University
- Floyd W. Bodyfelt, Co-Investigator, Dept. of Food Science and Technology, Oregon State University
- Miriam Martino, Post-doctoral Research Associate, Dept. of Food Science and Technology, Oregon State University
- Nilo Youssef-Hakimi, M.S. Graduate Student, Dept. of Food Science and Technology, Oregon State University
- Funding Sources:** Western Dairy Foods Research Center  
Centro Investigaciones en Criotecnología de los Alimentos (CIDCA), Argentina

### Objectives:

The goal of this research is to characterize the rheological and surface properties of a polysaccharide obtained by direct fermentation of whey, particularly acid whey. The specific objectives are:

1. To establish the fermentation parameters for the production of the polysaccharide produced by *Lactobacillus plantarum* 304 utilizing acid or sweet whey as the substrate.
2. To establish the procedures to recover and purify the polysaccharide
3. To analyze the fermentation broth by HPLC analysis and establish lactose utilization and yield parameters
4. To determine the surface tension properties of the unknown polysaccharide as affected by polysaccharide concentration, pH, temperature and ionic strength
5. To optimize fermentation parameters

## Results:

### a. Fermentation Studies

*Lactobacillus plantarum* 304, originally isolated from a cucumber fermentation, was routinely maintained and transferred in MRS (Difco, Inc.) broth medium. In all experiments, cultures of *L. plantarum* 304 were incubated without aeration. Acid whey was obtained from a local dairy plant and stored frozen until used.

Spontaneously occurring non-polysaccharide producing variants of *L. plantarum* can be isolated at a frequency of  $6.6 \times 10^{-3}$ . A method was developed to optimize differentiation of the colonies of the polysaccharide producing (poly+) and non-producing (poly-) strain using MRS agar followed by incubation at 25°C for 5-7 days. Polysaccharide producing strain showed larger, opaque and viscous colonies. Reversion to the poly+ phenotype was not observed.

Growth rate of both strains was measured using optical density (650 max. wavelength) at 20, 25, 30, 35 and 40°C. The results showed that growth rates were not significantly different between the two strains. Optimum temperature for the growth was 35-36°C and the detected minimum growth temperature was 15°C (Figure 1).

A modified MRS medium was prepared by using all the components of the commercial medium with the exception of the glucose. Different sugars were added to the medium as indicated by specific experiments. MRS media were heat sterilized and acid whey was filter-sterilized. Different sugars were examined as a sole carbon source for growth of the bacteria including glucose, fructose, galactose, lactose and sucrose at 2% concentration. Both strains showed growth utilizing these sugars; however, no growth was observed when ribose was the sole carbon source. Addition of calcium or magnesium to the media (at 2%) retarded the growth to some degree.

### b. Functional Properties Studies

Poly- cultures were used as negative controls along with uninoculated broths in viscosity measurements using Cannon-Fenske viscometers. Maximum viscosity was observed at about 24 hours of growth with a subsequent decrease with extended incubation (Figure 2). Polysaccharide was produced in modified MRS broth supplemented with either glucose, fructose, mannose, galactose, lactose, sucrose, cellobiose, maltose, trehalose, or raffinose as the sole sugar source. Ribose and arabinose produced no polysaccharide. Acid whey supported growth as well as polysaccharide production. The greatest yield of polysaccharide from acid whey occurred when the pH (4.3) was adjusted to pH 6 or 7.

A polysaccharide isolation and purification technique was also developed (Figure 2). Both polysaccharide isolation procedures gave a purified yield of about 3 g/l from MRS medium. The filtration method produced polysaccharide free of cells but required longer processing times. The yield of polysaccharide



produced at different incubation temperatures (18, 25, and 37°C) did not differ significantly. Samples were collected at the same pH value to obtain polysaccharide after equivalent fermentation levels.

The viscosity of the purified polysaccharide was determined using a Brookfield Viscometer (spindle series LV). Aqueous solutions of the isolated polysaccharide, 0.1 - 5% w/v, exhibited low viscosity (less than 10 cp). Viscosity increased slightly with polymer concentration. Attempts to stabilize the viscous behavior observed in the fermentation broth, e.g. by addition of divalent salts ( $\text{Ca}^{++}$ ), were only partially successful. All tested solutions exhibited a viscosity decrease with higher shear rates, typical of solutions with pseudoplastic behavior.

Preliminary studies have shown emulsion stabilization properties of oil/water mixtures. The samples with 1% w/v purified polysaccharide in water remained emulsified longer than samples with no polysaccharide. Microscopic examination showed smaller globules of the dispersed phase. These findings suggest that the polysaccharide could be used for emulsion stabilization. Initial and equilibrium surface tension measurements are being done to evaluate the use of the polysaccharide as a emulsifying agent not increasing food viscosity.

### **Impact of Research:**

A major problem faced by today's dairy industry is to find profitable uses for whey. In spite of new uses, whey utilization remains around 50%. The rest is discharged with a continuously increasing disposal cost. The production of polysaccharides with functional properties of commercial interest uses a waste to generate a by-product with commercial value.

The utilization of the polysaccharide to form natural coagulants for wastewater treatment was not considered as one of the specific objectives of the research effort. The use of the polycation (chitosan)-polysaccharide complex constitutes a new alternative to recover proteins from acid whey and other dairy waste streams. A treatment process could therefore involve the production of a "natural" emulsifying agent by fermentation of acid whey after recovery of the whey protein fraction using the complex as a coagulating agent. This process would utilize both the lactose and the protein whey fractions.

### **Publications and Abstracts:**

#### Presentations completed

Martino, M., and Torres, J.A. 1990. Better utilization of cheese whey. Presented at the 79th Annual Oregon Dairy Industries Conference, Feb. 13-14, Eugene, OR.

Martino, M., Youssef-Hakimi, N., Daeschel, M.A., and Torres, J.A. 1990. Food grade stabilizer produced by *Lactobacillus plantarum* from acid whey. Presented at the 79th Annual Oregon Dairy Industries Conference, Feb. 13-14, Eugene, OR.

Martino, M. and Torres, J.A. 1990. Natural chitosan complexes: an alternative for the treatment of processing wastewater. Presented at the Pacific Fisheries Technologists Meeting, Feb. 18-21, Portland, OR.

#### Upcoming presentations

Martino, M., Torres, J.A., Bodyfelt, F.W., and Daeschel, M.A. 1990. Characterization and isolation of a polysaccharide produced by *Lactobacillus plantarum* from acid whey. To be presented at the 51st Annual Meeting of the Institute of Food Technologists, June 17-20, Anaheim, CA.

Martino, M., Bouzas, J., and Torres, J.A. 1990. Recovery of suspended solids from food processing wastewaters using chitosan-polyanion complexes. To be presented at the Sixth International Symposium on Agricultural and Food Processing Wastes, December 17-18, 1990, Chicago, IL.

Bouzas, J., Martino, M., and Torres, J. A. 1991. The use of chitosan-polyanion complexes for the treatment of food processing wastewaters. To be presented at the 5th International Conference on Chitin and Chitosan, October 17-20, Princeton, NJ.

#### Publications

Martino, M., Torres, J.A., Bodyfelt, F.W., and Daeschel, M.A. 1990. Characterization and isolation of a polysaccharide produced by *Lactobacillus plantarum* from acid whey. In preparation.

Martino, M., Daeschel, M.A., and Torres, J.A. 1990. Caracterización fisicoquímica de un hidrocoloide obtenido a partir de la fermentación de suero de leche. In preparation.

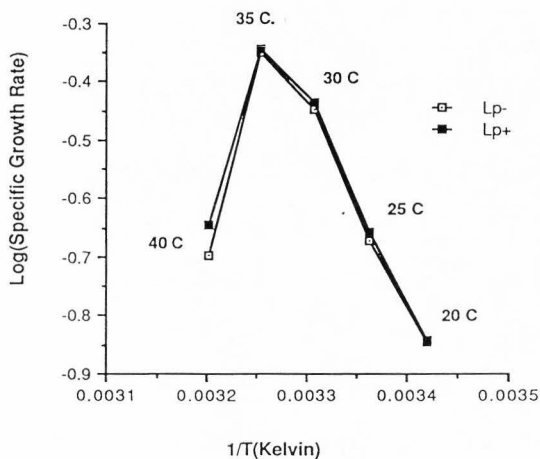
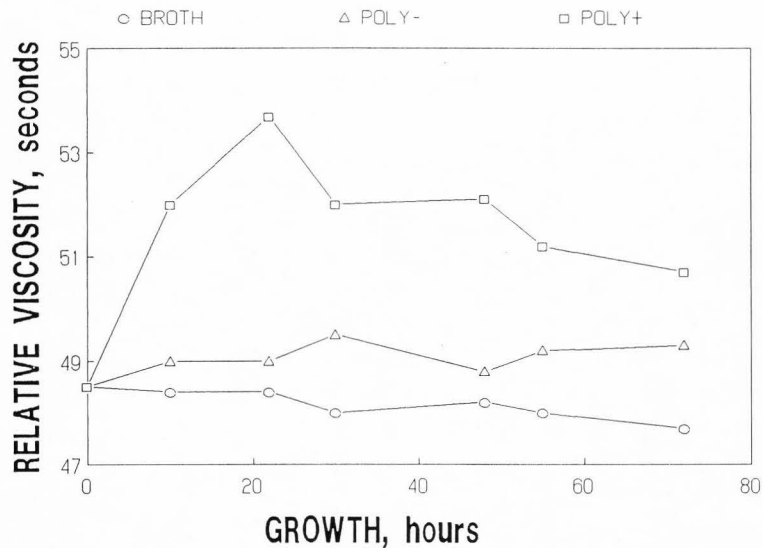


FIGURE 1- Arrhenius plot of growth rate of polysaccharide producing (Lp +) and non-producing (Lp -) strains of *Lactobacillus plantarum* vs. temperature

Figure 2. Viscosity Changes in MRS medium at 37C



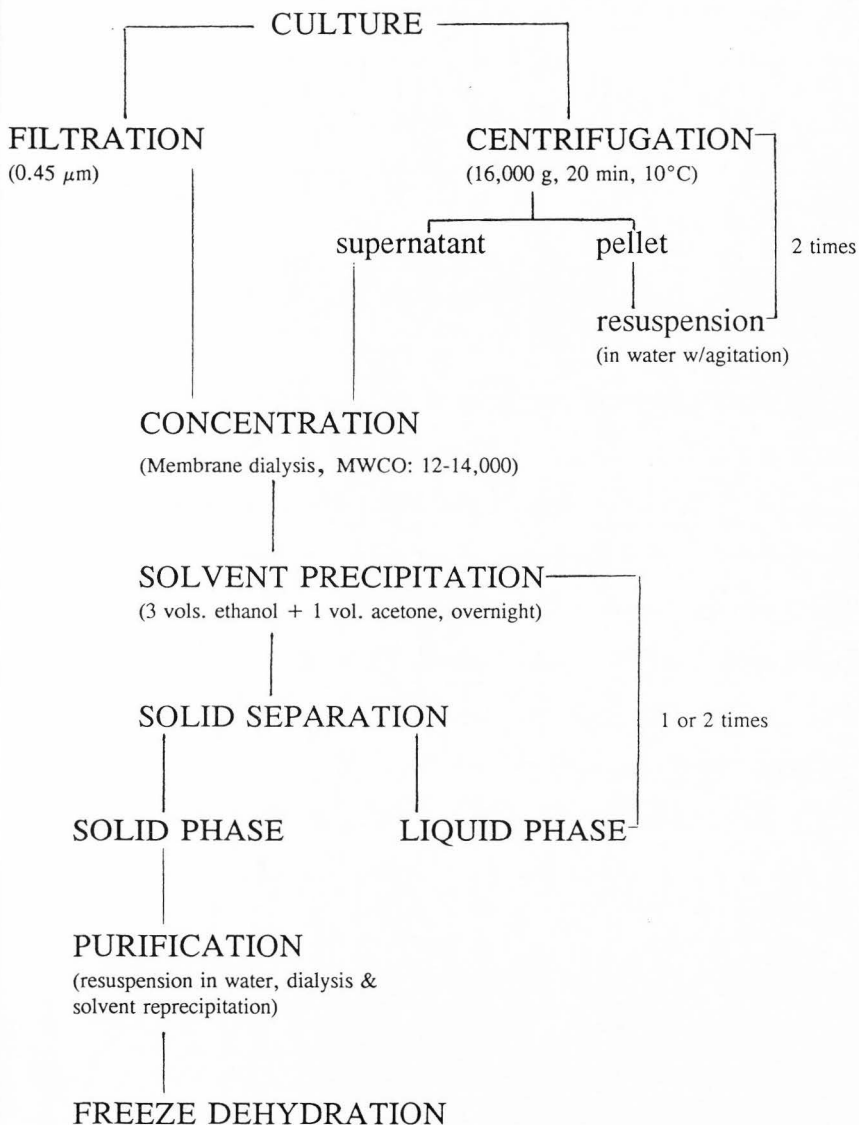


Figure 3. Polysaccharide Extraction and Purification Procedure

**Project Title:** Utilization of Acid Whey as a Substrate for the Production of Food Grade Cellulases

**Personnel:** Michael H. Penner, Dept. Food Science and Technology  
Oregon State University

Soren Nordmark, Dept. Food Science and Technology  
Oregon State University

**Funding:** Western Dairy Food Research Center

**Objectives:**

The use of enzymes in the food and related industries is growing rapidly due to decreases in the costs of producing relatively pure enzymes of high activity. The cellulase enzymes typify this trend as they are increasingly used in fruit juice processing and in the utilization of food processing wastes. One of the most expensive parts of industrial enzyme production is the cost of the substrate used to sustain the enzyme producing microbes. Alternative low cost substrates are, therefore, of interest to industrial producers. Whey products have the potential to be an attractive substrate for enzyme production. This is because whey products are rich in utilizable energy and several industrial microbes are capable of growing at relatively low pH.

The study is designed to test the feasibility of utilizing acid whey and whey permeate as primary substrates for the production of food grade cellulases by the mold *Trichoderma reesei*. A more general aspect of the study is to demonstrate that acid whey has beneficial properties that make it an attractive substrate for the production of secondary metabolites and enzymes by the general class of acid tolerant fungi.

**Results:**

Our initial studies have found that cellulase enzymes are produced by *Trichoderma* when grown on acid whey, pH 4.4. Enzyme yields were estimated to be approximately 50% of that observed for the same fungus grown under optimum conditions. The quantity of enzyme produced in different cultures is based on the traditional cellulase filter paper unit of activity. Adjustment of the whey substrate to pH to 5.0, 5.5, 6.0 and 6.5 prior to *Trichoderma* inoculation resulted in more reproducible enzyme production but only minimal increases in the median levels of enzyme produced. Soluble protein production at days 4 through 8 post inoculation has followed that of the control culture. The pH of all inoculated whey cultures increases by approximately 1 pH unit over the course of the 8 day incubation. This 1 pH unit rise has been consistent for whey preparations starting at pH 4.4, 5.5 and 6.5. Current studies are evaluating the use of an initiator to further stimulate cellulase production in the acid whey cultures. Studies evaluating the use of whey permeate as a substrate for *Trichoderma* cellulase production and simultaneous production of mycelial protein are also in progress.

## Impact of Research:

The optimum scenario for this research is that the results will clearly demonstrate that acid whey or whey permeate is a cost effective substrate that may/should be exploited by major enzyme suppliers. The fungus, *Trichoderma*, and the enzyme system, cellulase, which are the focus of this research should be considered exemplary, since acid whey may potentially be utilized by a range of fungi which are currently being used for the production of food grade enzymes. Other secondary metabolites, such as pharmaceuticals and pigments, are also potential products which could conceivably be produced from microbes growing on whey substrates. Consequently, this research will be of significance to the food, pharmaceutical and chemical industry in terms of demonstrating substrate properties of whey products. The impact to the dairy industry will be realized if industrial producers accept acid whey as a microbial substrate, thereby providing a mechanism for the utilization of currently underutilized acid whey.

## Ultrafiltration/Reverse Osmosis



*Cottage Cheese from Ultrafiltered Skimmilk by  
Direct Acidification*

*by*

*Jorge Ricardo Ocampo-Garcia*

*Master of Science*

*Utah State University, 1987*

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## ABSTRACT

Cottage Cheese from Ultrafiltered Skimmilk by  
Direct Acidification

by

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Pasteurized skimmilk at 4°C was acidified to pH 5.8 with 85.5% phosphoric acid (136g  $H_3PO_4$ /100 kg skimmilk), then warmed to 54°C and ultrafiltered to a protein concentration  $9.1 \pm 0.2\%$ . The retentate was heated to 76.5°C for 16 s then cooled to 2°C. Phosphoric acid (85.5%) was added at a rate of 3.41g per kg retentate. The acidified retentate was slowly warmed to 29.5°C (3°C/5 min) when the pH was checked. The pH at this point was no lower than 5.4. Heating was continued until a temperature of 32.2°C was reached. Glucono delta lactone was added to the retentate (17.6 g/kg retentate) and left undisturbed for approximately 80 min. The curd was cut at pH 4.7 with 0.64 cm curd knives and allowed 10 min for syneresis. Permeate obtained from the same lot of milk was acidified to pH 4.8 (66 g  $H_3PO_4$ /100 kg permeate), then added to the curd at 32.2°C (three parts permeate to four parts retentate) and used as a cooking vehicle. The curd was cooked to 59°C in 90 min. The curd was held at 59°C for 10 min, drained and washed once with ice water. Cream dressing containing 12.5% fat and 3% salt was

used at the rate of two parts curd to one part dressing.

Control cottage cheese was produced by a direct acid method from the same skimmilk used to produce ultrafiltered curd.

Use of ultrafiltered skimmilk retentate for cottage cheese making resulted in 2.24% more curd (corrected to 20% solids) and 2.24% more curd per kg original milk protein than the control. However, satisfactory firmness in UF curd required slightly more than 20% solids in the final product. Sensory evaluations indicated that creamed cottage cheese was not significantly different ( $p < 0.05$ ) from control cheese, but was better than commercial cottage cheese samples selected from the local market.

(69 pages)



## INTRODUCTION

The practical application of membrane ultrafiltration (UF) in the manufacture of different types of cheese was reported by Maubois and Mocquot (50).

Ultrafiltration of milk as part of the cheese making procedure continues to attract the attention of dairy researchers as well as the cheese industry. Ultrafiltration is now used in the manufacture of high moisture cheese varieties such as feta, camembert, and ricotta (50). Cottage cheese curd with 80% moisture should be a good candidate for using this process of concentration.

To be considered attractive for cottage cheese, the process must result in a product that is at least as acceptable as curd made by traditional processes. It also must provide some advantages over traditional processes with respect to increased yield and/or reduced cost of manufacture. Increased yield, vat efficiency and its potential application in continuous curd manufacture offer important possibilities for use of UF in cottage cheese making. Several applications of membrane processes for cheese making are found in the literature. In contrast, very little information is available on skim milk retentate used for cottage cheese manufacture.

Matthews, et al. (49) manufactured cottage cheese from retentate and suggested that a concentration ratio for skim milk of 1:2 (6.2% protein) was the apparent upper limit for obtaining a good quality product. They improved vat efficiency, but did not show any increased yield (49).

Studies with retentates (15% protein) by Covacevich and

Kosikowski (14) have shown the possibility of making ultrafiltered curd into whipped cream cottage cheese with improved color and appearance.

Possible yield increases of 12.4 and 15.3 from 16% total solids (TS) retentate (9.3% protein) from cultured and direct acid cottage cheese respectively, were reported by Narasimhan (58).

Raynes (65) found that cottage cheese curds made from 3 fold concentration (9.2% protein) retentates were not easy to cut with 0.64 centimeter or quarter-inch curd knives. Also after dressing was added, the texture became soft, pasty and sticky. These resulted in a gradual translucence during storage.

Texture and cream absorption by curd appear to be the principal problems in making cottage cheese from skimmilk retentates (14,49,58). Further success in making cottage cheese from skimmilk retentates depends upon solving problems of cooking curds and developing proper texture and cream absorption, (14). Covacevich and Kosikowski (14) reported consistently less whiteness, less acceptable appearance and tougher texture than conventional commercial cottage cheese when skimmilk retentate was used.

The purpose of this study was to develop a procedure for the manufacture of cottage cheese by direct acidification from preacidified, ultrafiltered skimmilk retentate (3 fold concentration), then compare the yield and quality of product with that of conventional cottage cheese.

## LITERATURE REVIEW

Cottage cheese is the soft, uncured cheese prepared by mixing cottage cheese dry curd with a creaming mixture. The milk fat content is no less than 4% by weight of the finished food (11).

Lowfat cottage cheese is the food prepared from the same ingredients and in the same manner as prescribed for cottage cheese. Its content of milkfat is not less than 0.5% and not more than 2% by weight. Its moisture content is not more than 82.5% (11).

Cottage cheese is conventionally made from skimmilk by the addition of starter culture and small amounts of rennet. However the use of starter cultures for acid production may involve difficulties such as agglutination of starter bacteria, lack of product uniformity, slow acid production, etc. (23). To eliminate these problems, direct acidification has been approved as an alternative (23).

Ultrafiltration is a process by which small molecules are separated from large molecules by passing a solution under pressure through a designated molecular weight cut-off membrane (30). Recently ultrafiltration has been applied to dairy products for concentration of whey protein, skimmilk protein, and as a first step in cheese making (51,30,60).

### Advantages of Membrane Ultrafiltration

One of the main advantages of ultrafiltration of milk for cheese making is increased cheese yield. This increase is due to improved retention of proteins and fat in the cheese (49).

This process also results in increased production per vat of

retentate which increases plant capacity with the same cheese making equipment. Ultrafiltration units occupy less floor space than cheese vats. This provides a substantial economy in investment for construction. Also, ultrafiltration equipment can be controlled automatically and offers potential application in continuous processes. Reduction in labor costs can be expected (49,32). Reduction in cost of 20% was reported when ultrafiltration was used for making feta cheese in comparison with conventional techniques (81). Chambers and Marks (9) reported that cottage cheese from ultrafiltration can reduce energy usage by 1614 Btu/kg of product when compared with standard techniques. Ultrafiltration on the dairy farm offers the ability to use permeate for animal feed, requires less holding tank capacity, uses less energy to keep the retentate refrigerated and reduces the cost of shipping retentates to cheese plants (91).

The economy of ultrafiltration is based, among other things, upon much lower rennet consumption than in conventional processes (39). Rennet requirements are inversely proportional to the degree of concentration and to the protein content of the milk. Rennet levels can be adjusted in order to maintain the usual clotting time (77). Hence, ultrafiltration may save up to 80% of rennet needed for making cheese.

Lack of uniformity in milk supply can be solved by using ultrafiltration. Standardizing the protein content in milk without detectable organoleptic consequence is one solution proposed by Poulsen (63).

The concentration of milk without chemical damage and flavor

changes caused by heating can be another advantage of UF in the dairy industry (30).

Pollution of cheese factory effluent is reduced due to less protein in permeate than in whey. Hence lower biological oxygen demand (30,48).

#### Cottage Cheese Making with Starter

Manufacture of cottage cheese with starter organisms involves the use of mixed strain acid-producers like Streptococcus lactis and Streptococcus cremoris and a flavor-producer, Leuconostoc citrovorum. Bulk starter is prepared by heating skim milk to 91°C for 30 to 60 min, cooling to 21°C, then inoculating with 1% stock starter culture. The inoculated skim milk is incubated at 21°C (23,70) until the pH is 4.6-4.8 and then refrigerated.

Different combinations of time, temperature and percent culture inoculum are used for cottage cheese manufacture. There is a short set method in which skim milk is inoculated with 5% culture and allowed to set for 4-6 h at 32°C. The long set method requires 12-16 h at 22°C with 1 to 2% inoculum. Each yields an equally high quality cheese curd and the selection of the method depends on the schedule of personnel in the plant (5,23,70). After cutting the curd, the cooking procedure is essentially the same for each method. The whey is drained and the curd washed to remove excess acid and lactose, and finally creamed (23).

The successful manufacture of cottage cheese with starter organisms depends on the activity of the bacteria culture (5,23). Lactic cultures are the source of most problems associated with the

traditional method (5,21,45,71). Some strains can agglutinate and precipitate out of skimmilk, causing sedimentation on the bottom of the vat and slow acid production (23). Agglutination of starter bacteria also results in a shattered and mealy curd. Lack of product uniformity due to variation in behavior of cultures also results (24). Proteolytic activity of some strains of starter bacteria affect the curd strength of cottage cheese and titratable acidity of whey (23,24). When improper combinations of lactic cultures with aroma bacteria are used, floating of curd and excessive foaming can occur during cooking (71). Lactic cultures can be inhibited by antibiotics and bacteriophage (23,45), reducing the production of acid. Growth of contaminating organisms during long setting periods may produce bitter flavors in the cheese. These problems make the traditional method difficult to control and to standardize.

#### Cottage Cheese Making without Starter

##### Chemical Acidification

A number of workers (19,25,53,80,85) have produced cheese by chemical acidification. Three approaches have been used.

- i) Direct addition of acid
- ii) Acidification by ester hydrolysis
- iii) Combination of direct acid and ester hydrolysis for acid production.

##### Direct Addition of Acid

Ernstrom (24) found that milk at refrigeration temperatures

could be acidified to the isoelectric point of casein without causing coagulation. He used hydrochloric acid because it was very inexpensive. Little (43) patented a process for cottage cheese production by coagulation of cold acidified milk with the addition of large amounts of rennet (10 to 66 ml/454 kg). Several patents were obtained (42,84,86,87) but the process had limited commercial application. One reason was, at that time, Federal laws did not permit the use of chemical acidulents in cheese making, and lower quality body and texture was found when compared with traditionally-produced cheese. However, Born and Muck (8) reported that consumer acceptance of cottage cheese made by direct acidification was not significantly lower than traditionally-produced cheese.

#### Hydrolysis of Acid Anhydrides

The selection of compounds which hydrolyze slowly to produce acids was investigated by Deane and Hammond (17). Glucono delta lactone (GDL) and meso-lactide were allowed to hydrolyze to their corresponding acids in undisturbed skim milk for the production of cottage cheese. The curd obtained with this method was smooth, uniform and very similar to that produced by starter culture (17). However, the use of these anhydrides was limited by their high cost and the time required for hydrolysis.

#### Combination of Direct Acid and Anhydride Hydrolysis

The use of liquid acids and acid anhydrides was used early for the production of Cheddar type cheese (46). Loter and Schafer (44) and Corbin (13) both patented a similar method in which phosphoric

acid and glucono delta lactone were used for acidification of skim milk in the production of cottage cheese. This process came to be the Vitex/American Direct set method (19). At present this method is used in several cheese plants with successful results (2,3,19,74). Actually about 30% of the cottage cheese in the United States is made using this method (Michael Gerson, Carlin Foods, St. Louis, Mo. Personal Communication, 1986).

#### Advantages of Direct Acidification

A major benefit of the direct acid process is the reduction in process time (27). Other benefits include more efficient equipment utilization, improved cheese consistency, improved process control and production rates, and the elimination of problems associated with culture growth (28). The yields obtained by this method are significantly higher than those obtained by culture methods (29,80). Geilman (27) showed that yield increases were due to better protein recovery. However there is some disagreement (89). Satterness et al. (73) did not find any significant difference in yield between the two processes. Some differences in manufacturing procedure and differences in the method of calculating yield may have contributed to different conclusions between the two methods of acidification (27). The differences could have resulted from 1) exclusion of weight of acidifying agents as part of milk solids, 2) use of cold skim milk to disperse GDL instead of cold water, 3) differences in moisture adjustment of the two curds. Reduced yield in cultured cottage cheese may have been due to bacterial proteinase activity and to changes in the proportion of Prt+ vs Prt- cells during



culture maintenance (67).

#### Disadvantages of Direct Acidification

Texture and body of direct acidification (DA) cottage cheese have sometimes been found inferior to traditional cottage cheese (25,73,85,89). However, quality of direct acid cottage cheese is not significantly different from traditional cultured cheese (8,27). Prices of ingredients per kilogram of cheese for DA were higher than costs of traditional culture methods of acidification. Nutritional quality has been considered. McDonough and Alford (52) found no significant difference between protein efficiency ratio (PER) of DA versus traditional cottage cheese, although the vitamin content was slightly higher. Other workers (69,82) found no significant differences in calcium, magnesium, phosphorous and iron retention between the two types of cheese.

#### Heat Treatment of Skimmilk

Increasing cottage cheese yields by heating skimmilk to temperatures where whey proteins are denatured and included in the curd, has attracted the attention of the dairy industry. Normal pasteurization temperatures for skimmilk are 61.7 to 62.8°C for 30 min or 71.7 to 72.2°C for 15 s (23). At higher temperatures (80°C) denaturation of whey proteins occurs (33). Excessive heating of skimmilk results in a soft curd that breaks when cut, and does not reach the desired firmness when cooked. Serum protein denaturation greater than 10% with heat treated skimmilk is not satisfactory for cottage cheese manufacture (57).  $\beta$ -lactoglobulin is the most abundant whey protein. During heat treatment  $\beta$ -lactoglobulin

denatures above 80°C, due to disulfide interchanges. Destabilization of the residual protein structure occurs near 140°C (88). The rate of heat denaturation depends on pH.  $\beta$ -lactoglobulin is most stable in pH range of 5.0 to 7.0 with a maximum at pH 6.0. It is most sensitive to heat at pH 4.0 (18). At high temperatures,  $\beta$ -lactoglobulin and k-casein interact with each other resulting in loss of solubility (76).  $\alpha$ -lactalbumin is the smallest and most heat-resistant whey protein. The cysteine residues are mainly responsible for its stability (18). However during heat treatment  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin interact and form a complex with k-casein. Some  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin will pass through the ultrafiltration (UF) membrane during concentration (6,61). Heat treatment may decrease these losses.

Heating skim milk to 79.4°C for 30 min results in 10% greater curd yield than when heating is limited to pasteurization. This is due to heat denaturation of the whey protein and its inclusion in the curd. However, the finished product is usually mealy. Also the pH of the cheese is higher than normal because the pH at the acid coagulation (AC) endpoint (5.2) (best pH to cut cottage cheese curd) is higher than the AC endpoint in unheated milk (22).

White and Ray (89) reported the manufacture of cottage cheese by continuous fermentation using different heat treatments (73.8°C/17 s; 100°C/7.9 s; 120°C/7.9 s; 135°C/3.9 s) of skim milk. Increasing heat treatments resulted in high moisture in the curd and long cooking times. Low temperatures (73.8°C and 100°C) gave the best body and texture scores. At constant moisture levels, the two

lower temperatures showed slightly greater cheese yields. However pasteurizing the skimmilk at 100°C for 7.9 s resulted in the best yield.

#### Heat Treatment of Retentate

The effect of heat on concentrated skimmilk for cottage cheese making was studied by Emmons et al. (22). Pasteurized (60°C/30 min) skimmilk was divided into four lots and concentrated to 47.6, 43.5, 43.9 and 40.1% total solids (TS). Each concentrated lot was divided into five portions; one portion was used as a control and the other four portions heated to 54.4, 60, 65.6 or 71.1°C for 15 min, cooled and diluted to 8.8% TS, for cottage cheese manufacture. They did not observe any significant difference in the amount of undenatured whey proteins. However they reported that the control had the best curd quality. Heating the concentrated skimmilk to 71.1°C for 15 min had a deleterious effect on curd quality (mealy and soft) (22). Increasing TS generally limits the effect of heat on the rate of protein denaturation (37). Increase in ionic strength and low pH decreases  $\beta$ -lactoglobulin denaturation in skimmilk concentrate (92). Green et al. (31) observed that when skimmilk was acidified to pH 6.0 before ultrafiltration (1 to 4x) it gelled under all heat treatment conditions used (100°C and 119°C for 15 min and 140°C for 4 s). However they established that less denaturation of whey proteins occurs when the milk is concentrated (31). Birkjaer, (7) suggested that heating retentate to 77°C for 1 min can reduce bacterial counts and improve the body and texture of cheese. The use of higher temperatures (90°C - 95°C) reduces cheese quality.

Maubois and Mocquot (50) observed the restoration of curd-forming ability in ultra-high temperature (UHT) milk concentrated by ultrafiltration. Similar results are obtained when retentate prepared by UF of normal milk is treated by UHT (50). Shammet (79) reported that 76.7°C for 16 s was the best heat treatment for preacidified retentate (38% TS) pH 6.0, to make white soft cheese (Middle Eastern type). Extending the heating time of retentates increased mealiness of the finished product. This does not support Anis and Ernstrom (1) who stated that heating retentates (40% TS) at 82.2°C for 30 min improved the texture and the body of Domiati cheese.

#### Cottage Cheese from Ultrafiltered Skimmilk

Maubois and Mocquot (51) used skimmilk retentate (27% TS) in combination with cream and obtained a product with the same composition as a soft cheese. Matthews et al. (49) used retentate (6.4% protein) from skimmilk to make cottage cheese. They found no difficulty in curd formation, however, they observed slow acid production which was attributed to lactic started culture. After cutting, agitation was difficult to initiate without matting the curd. Flavor and texture scores were not significantly different from the control. Total solids in the curd were 21-23%. When yields were adjusted to 20% TS, yield increases of 3.07, 4.87 and 5.86% greater than control were obtained. They concluded that yield improvement per vat was the major advantage (50% increase). The extent of UF was limited by the amount of curd that could be handled in the vat without matting during cooking. In this study, whey proteins were expelled from the curd during cooking resulting in

potential yield losses (49).

Covacevich and Kosikowski (14) produced cottage cheese from fermented retentates (15% protein). Yields were 7.8 and 2.3% higher than conventional cottage cheese (control). The experimental cheese curd had a lighter color, gelatinous appearance and absorbed cream poorly. These defects were attributed to high pH. By making ultrafiltrated curds into whipped cream, cottage cheese color and appearance improved. Proper texture and cream absorption need to be improved before making cottage cheese from high protein retentates (5x).

Narasimhan (58) reported increased yields over cultured cottage cheese (control) made from skimmilk when retentate contained 16 and 20% T.S. The yields were 12.4, 15.3, 5.6, and 1.6% for 16% cultured, 16% direct acid, 20% cultured and 20% direct acid cottage cheese respectively. This was attributed to entrapment of whey proteins in the curd and reduced syneresis of whey. Lower yield increases from 20% retentates were due to shattering of curd during cooking, and the diffusion of the whey proteins (58). Cultured cottage cheese from retentates was as good as that obtained from skimmilk. Direct acid cottage cheese was significantly lower in quality. Problems with lactic culture growth in retentate and the use of nonconventional cooking methods must be solved to obtain maximum benefit from UF.

Raynes (65) found that cottage cheese curds made from heat-treated retentates were not easy to cut with 0.64 cm knives. The cheese had a good texture and body, however a gradual translucency developed in the curd during storage. The texture became soft and

pasty and the UF curd absorbed all the dressing causing a sticky appearance.

Recently Kealey and Kosikowski (38) used retentate to supplement skim milk to produce industrial cottage cheese of comparable quality to cheese made from unsupplemented control skim milk.

#### Lactic Fermentation of Ultrafiltered Skim Milk

Many workers have observed the adverse effect on lactic cultures when concentrated skim milk and retentates are used as growth media (40,41,49,58). Cox (16) studied the growth of S. Lactis, S. diacetylactis, and S. cremoris strains in concentrated milk and found that growth of all strains was inhibited when TS exceeded 36% (12.96% protein). Pulay and Krasz (64) observed an increase in acid production by mixed starter cultures when total solids were increased from 28 to 32% (10.08 to 11.52% protein), but decreased above that level. Collins (12) intended to make cottage cheese from skim milk fortified to 12, 15 and 18% TS (4.32, 5.40, and 6.38% protein) with nonfat dry milk. In skim milk containing 15 and 18% TS, the pH decreased very slowly after 5.0 when 6% culture was used. pH changes are slower when retentate is used rather than normal milk. Longer incubation periods are required (15,55). As a consequence, retentates demand more starter bacteria for lactic acid production and more time during cheese manufacturing (35,54). Hickey et al. (35) used retentate (5x) and observed an increased concentration of lactic acid with minimum change in pH. Apparently UF caused stimulation of growth and acid production above their

normal levels in a milk substrate. Buffer capacity is due to protein concentration, insoluble calcium and phosphate salts. Large amounts of acid are required to lower the pH in retentates (54). When milk is concentrated to 5x, its buffer capacity is seven times higher than normal milk (54). Mistry and Kosikowski (56) measured pH change during fermentation of UF retentate with lactic cultures. The pH resisted change below pH 5.2. Even after 8.5 h the pH did not reach 4.6 while control required 6 h.

Narasimhan and Ernstrom (59) also reported slow acid production when retentate was used in cottage cheese manufacture. They attributed the problem to the high concentration of colloidal calcium phosphate (90). Addition of phosphate to skim milk slows lactic cultures, and the removal of phosphate from retentates enhanced acid production. Soluble phosphates were major factors in inhibiting acid production by lactic cultures. This explains the inhibition encountered below pH 5.0 when colloidal calcium phosphate is solubilized.

Recently Pope (62) stimulated Streptococcus cremoris UC310 by adding 0.02% yeast extract to 5x retentate and reduced the time to reach pH 5.1 from 24 to 10 h. In addition, he observed that preacidification of milk to pH 5.8 prior to ultrafiltration reduced the demand for high acid production by cultures during cheese making.

## MATERIALS AND METHODS

Skimmilk

Raw whole milk was obtained from Utah State University Dairy Farm. This milk was separated and the skimmilk pasteurized at 63°C for 30 min and divided into two lots. One lot was ultrafiltered to  $9.1 \pm 0.2\%$  protein and made into cottage cheese. The other was made directly into cottage cheese. Both lots were stored at 2°C until used.

Manufacture of Cottage Cheese (control)

Skimmilk at 2°C was acidified with phosphoric acid (Vitex 750) to pH 5.5 following the Vitex Direct set method (19). The factor (0.00893308)(kilograms of skimmilk) was used to determine total kilograms of acid to add. The phosphoric acid was diluted five times with water. The acidified skimmilk was slowly stirred and warmed (3°C/5 min) to 32.2°C.

Rennet and glucono delta lactone were calculated using the following factors (19):

$$\begin{aligned} \text{kilograms of GDL (powder)} &= (\text{kilograms of skim milk}) \\ &\times (0.011525462) \end{aligned}$$

$$\text{milliliters of rennet} = (\text{kilograms of skim milk})(0.00639)$$

Rennet and GDL were diluted five times with ice water and added to the acidified skimmilk, agitated for no more than 5 min and allowed to stand at 32.2°C for exactly one hour before the curd was cut with 0.64 cm curd knives. The curd and whey were gently stirred while heating to a temperature of 54.5°C over a period of 1 h 30 min



or until a desired firmness was reached. The whey was drained and the curd washed with an equal amount of ice water as the whey drained, agitated for about 5 min and drained.

Cream dressing containing 12.5% fat and 3% salt was prepared according to Manus (47) and used at the rate of two parts curd to one part dressing.

### Manufacture of Cottage Cheese from Ultrafiltered Skimmilk

#### Manufacture Procedure

Skimmilk was acidified to pH 5.8 prior to ultrafiltration with 85.5% phosphoric acid (136 g  $H_3PO_4$ /100 kg skimmilk). Acidification was at 4°C to prevent localized coagulation of protein.

Ultrafiltration was by a batch method using an Abcor HFK-130 single stage, spiral wound, polysulfone membrane with a molecular weight cut-off of 10,000 daltons with 5 m<sup>2</sup> of filtering surface (Figure 2). A balance tank and centrifugal pump were used for recirculation. An inlet pressure of 420 kPa (60 psi) and outlet pressure of 280 kPa (40 psi) were used throughout the process.

Protein content in the retentate was adjusted to  $9.1 \pm 0.2\%$  by adding permeate from the same lot of milk.

Membranes were cleaned as follows: water rinse; alkaline wash (NaOH, pH = 11.5) and chlorine (300 ml/80 lt of water) for 30 min; water rinse; acid wash ( $HNO_3$ , pH = 1.5) for 30 min; water rinse. Equipment was sanitized immediately before use with water containing 200 ppm chlorine. Deionized water at 54.5°C was used for washing and sanitizing the ultrafiltration membrane.

Figure 1. Manufacture of cottage cheese from ultrafiltered  
skimmilk by direct acidification.

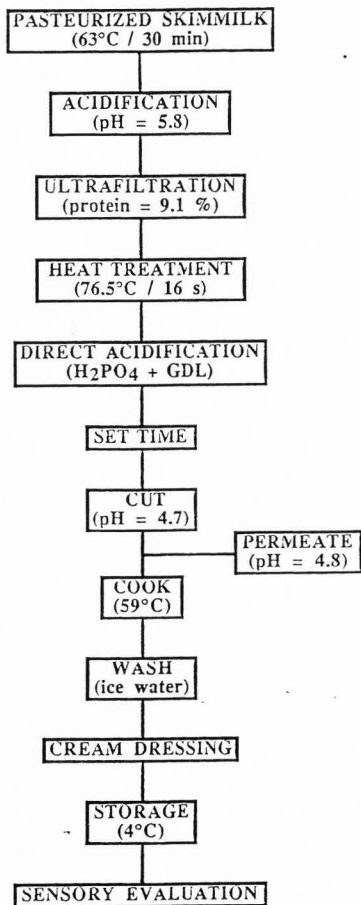
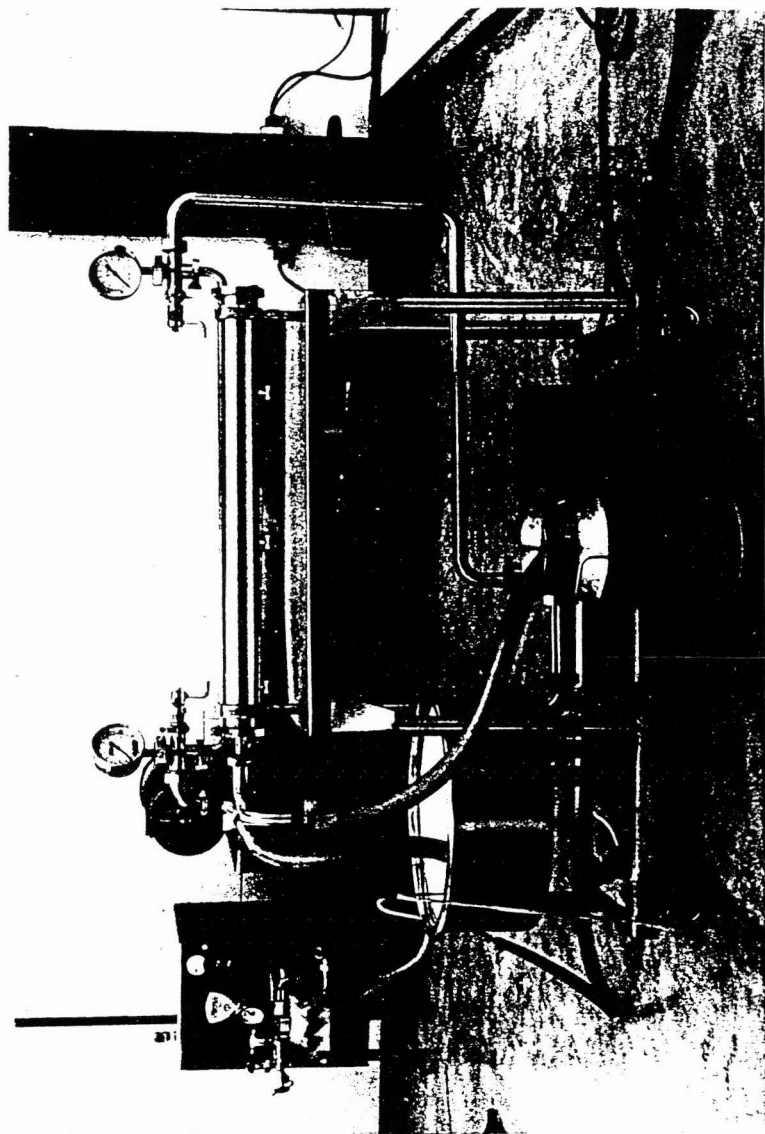


Figure 2. Ultrafiltration unit



### Heat Treatment of Retentate

A series of preliminary experiments were performed to study the effect of heat treatment of retentate on firmness of curd at cutting, and texture and body of the final product.

A high temperature, short time, plate heat exchanger (Pasilac Therm A/S KD 6000 Kolding, Denmark) consisting of a regeneration unit and heating section heated by circulating hot water from a water bath controlled by a steam thermo device, was used.

Retentate was heated at 65, 72.5, 78.5 and 80°C for 16 s and used for cottage cheese manufacture. Curd from the 65 and 72.5°C treatment was too tough to cut with knives. Curd from the 78.5 and 80°C treatment was too weak and soft. The temperature which resulted in the best cutting curd was 76.5 for 16 s.

### Cheese Making Procedure

Retentate was acidified at 2°C with 85.5% phosphoric acid (341 g per  $H_3PO_4$ /100 kg retentate) diluted five times with water and stirred continuously for 5 min. The acidified retentate was slowly warmed to 29.5°C (3°C/5 min). The pH at this point was no lower than 5.4. This was necessary to prevent precipitation of casein. Heating was continued until a temperature of 32.2°C was reached. Glucono delta lactone was diluted five times with ice water and added to the retentate (1.76 kg/100 kg retentate), stirred for no more than 5 min and left undisturbed for approximately 80 min. The curd was cut at pH 4.7 with 0.64 cm curd knives and allowed to syneresis for 10 min. Permeate (32.2°C) obtained from the same lot of milk was acidified to pH 4.7 (66 g  $H_3PO_4$ /100 kg permeate), then

added to the curd (three parts permeate to four parts retentate), as a cooking vehicle. The curd was cooked to 59°C in one hour 50 min according to the following rate:

<u>Time</u>	<u>Temperature (°C)</u>	<u>Increase (°C/5 min)</u>
0:00 - 0:30	32.2 - 35.5	0.5
0:30 - 1:00	35.5 - 42.2	1.1
1:00 - 1:50	42.2 - 59.0	1.6

The curd was held at 59°C for 10 min then drained and washed once with an amount of ice water equal to the whey drained.

The dressing was prepared and added to the curd as described for the control.

Creamed cottage cheese from UF skimmilk and control were packaged in 0.5 kg containers and stored at 4°C for one week before compositional analysis and sensory evaluation.

#### Composition Analysis

##### Moisture

Moisture was determined on 2.5 to 3.0 g of skimmilk or permeate, or 2.0 to 2.5 g of retentate or curd. Samples were weighed in an aluminum pan, evaporated on a steam bath, and dried for three hours at  $100 \pm 2^\circ\text{C}$  in a forced draft oven (Thelco model 28 - GCA Precision Scientific) (66). All samples were cooled in a glass desiccator prior to final weighing. Moisture determinations were in triplicate.

Samples revealing discrepancies were repeated until close agreement was achieved.

### Fat

Fat was determined by the Mojonnier modification of the Roesse-Gottlieb method (26) using samples of approximately 10 g for milk and permeate and 2.5 g for retentate and cheese.

Method of homogenizing samples before weighing depended upon sample consistency. Skimmilk and permeate samples were warmed and mixed in plastic bags. Retentate samples were warmed and mixed with a spatula. Cheese samples were chopped and mixed with a blender.

### Protein

Nitrogen was estimated by semi-micro Kjeldahl procedure (36) using automatic Kjeltex equipment (Kjeltex Auto 1030 Analyzer, Tecator, Inc.). Determinations were in triplicate and protein content was calculated by multiplying the nitrogen content of the sample by 6.38. Non-casein nitrogen determinations were by the procedure of Rowland (68) with some modification. Filtration was with Whatman No. 4 filter paper followed by Gelman 0.2  $\mu$ millipore filter paper to ensure that the filtrate was totally devoid of precipitated casein. Casein nitrogen was calculated as the difference between total nitrogen and non-casein nitrogen. Whey protein nitrogen was determined as the difference between total nitrogen and the nitrogen from casein and non-protein nitrogen.

### Lactose

Lactose was estimated by Shaffer-Somogyi method (78) using 2 g samples and expressed as percent anhydrous lactose.



### Calcium

Calcium was determined by atomic absorption (AA) spectrometry using a AA model 457 AA/AE spectrophotometer (Instrumentation Laboratory Inc.) (4).

Samples were digested by wet ashing (10). Ten milliliters concentrated nitric acid (16 M) was added to 1 g of all samples and digested for 48 h at 100°C or until a clear pale yellow solution was obtained.

Salts were dissolved in distilled, deionized water and made to 50 ml. The samples were diluted with 1000 ppm lanthanum oxide solution to bring the calcium concentration into the linear range of the spectrophotometer and reduce AA interference (75).

### pH

pH values of skim milk were determined before and after acidification, and at the time of UF. pH of retentate, permeate and cheese were also determined. A glass electrode and potentiometer (Model 811, Orion Research, Cambridge, MA 02139) were used for pH measurements.

### Sensory Evaluation

Cottage cheese from retentate, control and three selected commercial brands (one directly set and two cultured) were evaluated by a trained panel of four judges after one week of storage. The judges evaluated the finished product for flavor, appearance/color, body/texture and overall, using a grading scale of 1 = poor, 2 = fair, 3 = good, 4 = very good and 5 = excellent.

### Statistical Analysis

The taste panel parameters were analyzed by repeated measurement design with blocking across time to determine sources of variation (72). Treatment means were compared by the Least Significant Difference (LSD) method (20) for those treatments which had significant F-ratios.

## RESULTS & DISCUSSION

### Manufacture of Cottage Cheese

The most frequent problem encountered during the manufacture of cottage cheese from 3x retentate (9.2% protein) is the formation of a very tough curd at pH 4.7. The curd was not cuttable with conventional curd knives. This problem was reduced by heat treatment of the retentate prior to cheese making. The heat treatment was very critical because too much heat destroyed curd formation and resulted in excessive loss of fines. Too little heat left the curd too tough to cut. A heat treatment of 76.5°C for 16 seconds was found to be most suitable. Variations of as much as 2°C either way caused problems. Use of rennet made the curd even more tough to cut. Acidification of the skim milk to pH 5.8 prior to ultrafiltration corrected the problem of translucent and sticky curd after addition of cream dressing (R. Raynes, Utah State University, Personal Communication, 1984) and helped in the production of good quality cottage cheese curd from retentate.

The adjustment of retentate by protein instead of TS helped to control closely the amount of phosphoric acid and GDL that was used with this method and, consequently, setting time of the retentate and the final cook temperature were similar when different skim milk was used.

Acidification with phosphoric acid to a pH lower than 5.4 resulted in protein precipitation. Hence decreased curd strength increased amount of fines during cooking and a soft texture was observed in the final product.

The final curd was smooth and firm during cutting. Hence it was difficult to cut curd with conventional curd knives. In this study, cooking of the curd to temperatures in the range of 58.5 to 59.5 was critical in order to achieve the desired final texture of cottage cheese. Curds cooked to a final cook temperature below 58.5°C were judged too soft and weak; curds cooked to temperatures over 59.5°C were found to be too firm and mealy.

#### Composition Analysis

The composition of skimmilk used in this study is shown in Table 1. Total solids varied from 8.62 to 9.0%, protein from 3.16 to 3.27%, fat from 0.15 to 0.76%, lactose from 4.48 to 4.62% and calcium from 0.129 to 0.130%.

Mean composition of uncreamed cottage cheese is shown in Table 2. Uncreamed cottage cheese made from UF retentate was lower in moisture than the corresponding control curd in all three trials. This reflected the fact that it was necessary to cook the UF curd to 59°C in order to obtain satisfactory firmness, while the control curd was satisfactorily cooked at 57.2°C. Some preliminary experiments indicated that if cooked to less than 59°C, UF curd was still less than 80% moisture and lacked the desired meatiness of good curd. As a result of this moisture difference the percent protein in the UF curd was higher than in the control.

When all the curd was corrected to 80% moisture as shown in Table 3, there was no significant difference in protein between the UF and regular curd. Calcium content was significantly higher ( $p < 0.2$ )

Table 1. Composition of skim milk

		Trial I	Trial II	Trial III
%				
Total solids	AV	9.00	8.72	8.62
	SD	0.008	0.02	0.04
Protein	AV	3.27	3.25	3.16
	SD	0.02	0.03	0.01
Fat	AV	0.76	0.15	0.26
	SD	0.009	0.009	0.008
Lactose	AV	4.48	4.62	4.58
	SD	0.05	0.02	0.04
Calcium	AV	0.129	0.131	0.130
	SD	0.0001	0.002	0.004

AV = Average

SD = Standard Deviation

Table 2. Mean composition of uncreamed cottage cheese

%		Control	UF	LSD
Moisture	AV	78.93	76.98	N.S.
	SD	0.87	0.29	
Protein	AV	17.22	19.04	1.10
	SD	0.60	0.22	
Fat	AV	1.19	1.13	N.S.
	SD	0.18	0.11	
Lactose	AV	1.29	1.58	N.S.
	SD	0.11	0.35	
Calcium	AV	0.048	0.087	0.01
	SD	0.007	0.005	
Whey protein	AV	0.5755	0.6687	N.S.
	SD	0.035	0.006	

AV = Average

SD = Standard Deviation

Table 3. Mean composition of uncreamed cottage cheese  
(adjusted to 80% moisture)

%		Control	UF	LSD
Moisture	AV	80.00	80.00	-
Protein	AV	16.99	18.15	N.S.
	SD	0.42	0.13	
Fat	AV	1.17	1.09	N.S.
	SD	0.17	0.11	
Lactose	AV	1.27	1.52	N.S.
	SD	0.12	0.33	
Calcium	AV	0.048	0.084	0.040
	SD	0.006	0.005	
Whey protein	AV	0.546	0.594	N.S.
	SD	0.028	0.017	

AV = Average

SD = Standard Deviation

than in control. Matthews et al. (49) reported higher concentration of calcium in uncreamed UF cottage cheese (2x) than regular cheese. He attributed it to less solubility of ionic calcium at higher temperature (49°C) which resulted in association with colloidal milk proteins. However, protein, fat, whey protein and lactose in both UF and control cheese were not significantly different ( $p < 0.05$ ).

The composition of both creamed UF and control cottage cheese (Table 4) met U.S. legal standards of identity (11).

#### Cottage Cheese Yield

Since it was impossible to recover all the retentate from the small batch ultrafiltration unit, it was necessary to determine the amount of available retentate by using protein as a reference constituent. Protein in the original milk minus the protein in the permeate and permeate rinse water represented the protein in the retentate. By testing the protein content of the retentate, it was possible to compute the amount of retentate that should result from the UF process. The results of three trials are illustrated in Table 5.

Because of the difficulty of getting good UF curd with 20% TS, it was necessary to adjust actual yields to a constant total solids of 20% for adequate yield comparison. Actual and adjusted yields are shown in Table 5. Actual yields of UF cheese were 0.7, 7.8, and 10.4% less than the control, while curd adjusted to 80% was 1.71, 2.43 and 2.5% greater than the control. Also, the amount of cheese (adjusted to 80% moisture) produced per kilogram milk protein



Table 4. Mean composition of cottage cheese

%		Control	UF
Moisture	AV	77.93	76.98
	SD	1.17	0.29
Protein	AV	12.69	13.76
	SD	0.08	0.41
Fat	AV	5.34	5.13
	SD	0.77	1.03
Lactose	AV	2.10	2.28
	SD	0.34	0.34
Calcium	AV	0.069	0.095
	SD	0.006	0.008

AV = Average

SD = Standard Deviation

Table 5. Cottage cheese yield

	<u>Trial I</u>		<u>Trial II</u>		<u>Trial III</u>	
	<u>Control</u>	<u>UF</u>	<u>Control</u>	<u>UF</u>	<u>Control</u>	<u>UF</u>
Milk (kg)	186.14	186.14	181.83	181.83	180.01	180.01
protein (%)	3.27	3.27	3.25	3.25	3.16	3.16
protein (kg)	6.09	6.09	5.91	5.91	5.69	5.69
Permeate + rinse (kg)		123.17		118.95		124.17
protein (%)		0.26		0.26		0.29
protein (kg)		0.32		0.30		0.35
Retentate (kg)		62.58		61.55		59.40
protein (%)		9.22		9.11		9.02
protein (kg)		5.77		5.61		5.34
Actual Yield	14.20	14.01	14.60	13.46	14.60	13.08
% Total Solids	22.12	22.73	21.09	23.41	20.00	22.91
Adjusted Yield (20% TS)	15.70	15.97	15.40	15.77	14.60	14.98
% increase		1.71		2.43		2.59
kg curd/kg protein	4.802	4.884	4.736	4.850	4.62	4.74
% increase		1.71		2.41		2.60

was significantly higher ( $p < 0.05$ ) in UF than control cottage cheese.

### Sensory Analysis

#### Flavor

Mean flavor scores are shown in Figure 3. At  $\alpha = 0.05$  level, there were no significant differences among the cheeses. There were significant differences among judges and trials. UF cottage cheese had a very good flavor quality, as did the control cheese.

#### Appearance and Color

Mean appearance and color scores are shown in Figure 4. At  $\alpha = 0.05$ , no significant difference was observed among judges. However, there were significant differences among cheese samples and trials. No significant difference was observed between control and UF cheeses which were judged very good. The UF cottage cheese was graded better than two of the three commercial samples and no different than the third.

#### Body and Texture

Mean body and texture scores are shown in Figure 5. At  $\alpha = 0.05$ , no significant differences were observed among judges. However, there were significant differences among cheese samples and trials. No significant differences were observed between UF and control cheeses which were judged very good. The UF cottage cheese was better than two of the three commercial samples and equal to the third.

Figure 3. Means for flavor of cottage cheese samples: control, ultrafiltered, brand A directly set, brand B directly set and brand C cultured skimmilk. Bars with the same letter are not significantly different ( $p < 0.05$ ). Quality code is: 1 = poor, 2 = fair, 3 = good, 4 = very good, and 5 = excellent.

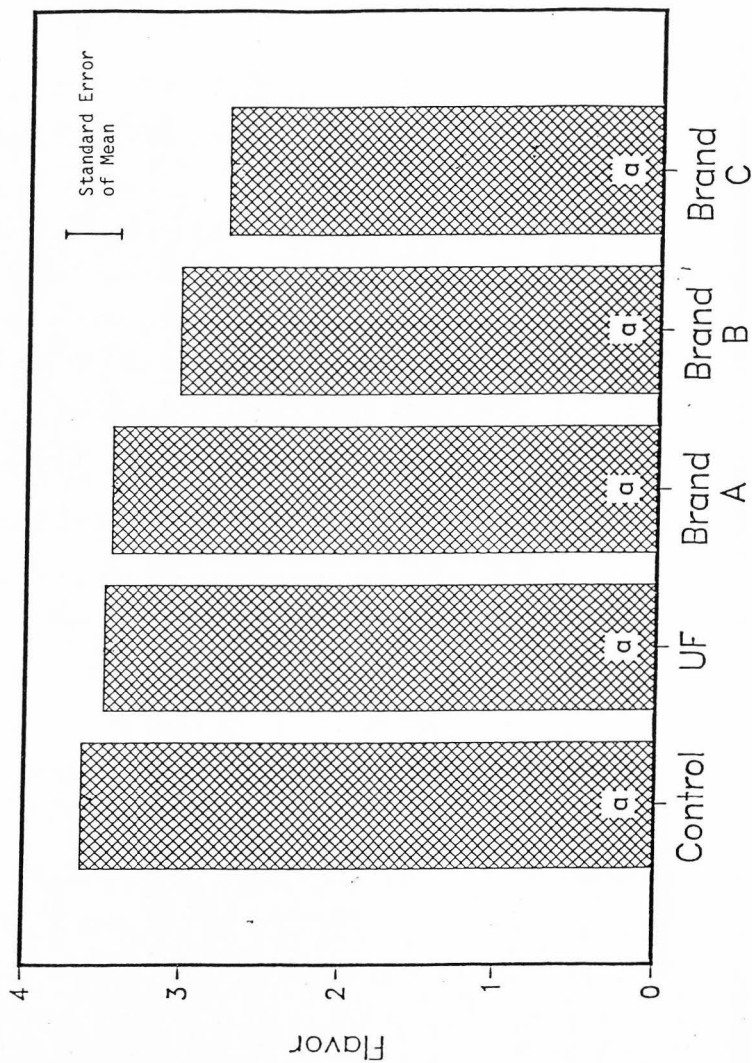


Figure 4. Means for appearance and color of cottage cheese samples: control, ultrafiltered, brand A directly set, brand B directly set and brand C cultured skimmilk. Bars with the same letter are not significantly different ( $p < 0.05$ ). Quality code is: 1 = poor, 2 = fair, 3 = good, 4 = very good, and 5 = excellent.

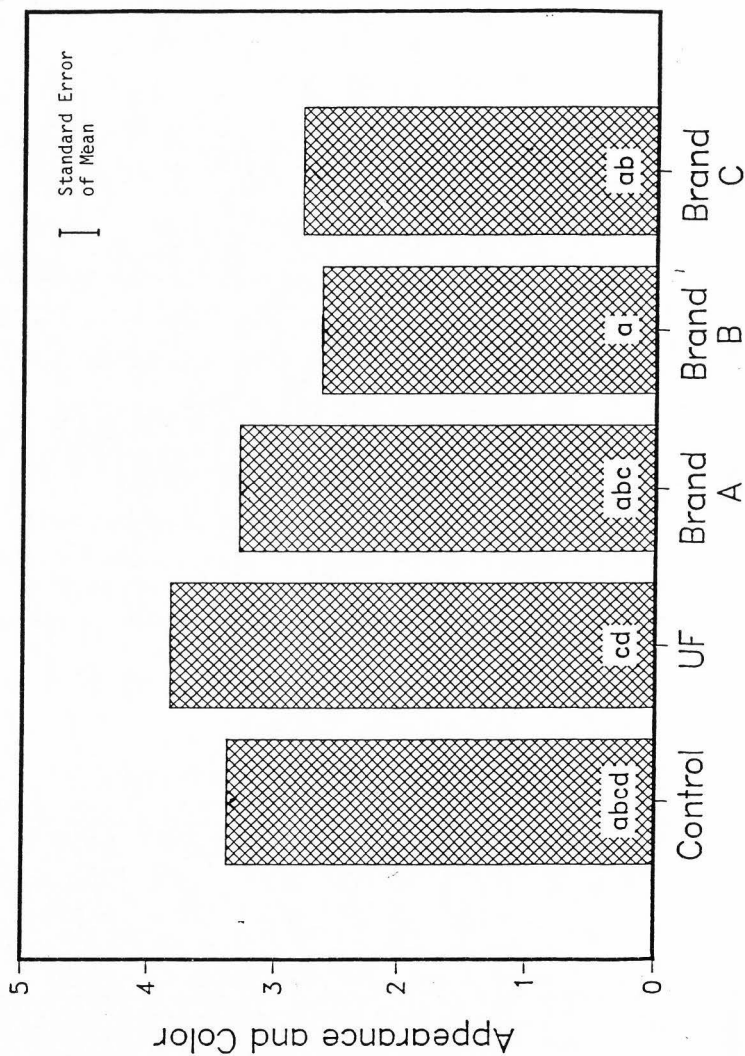
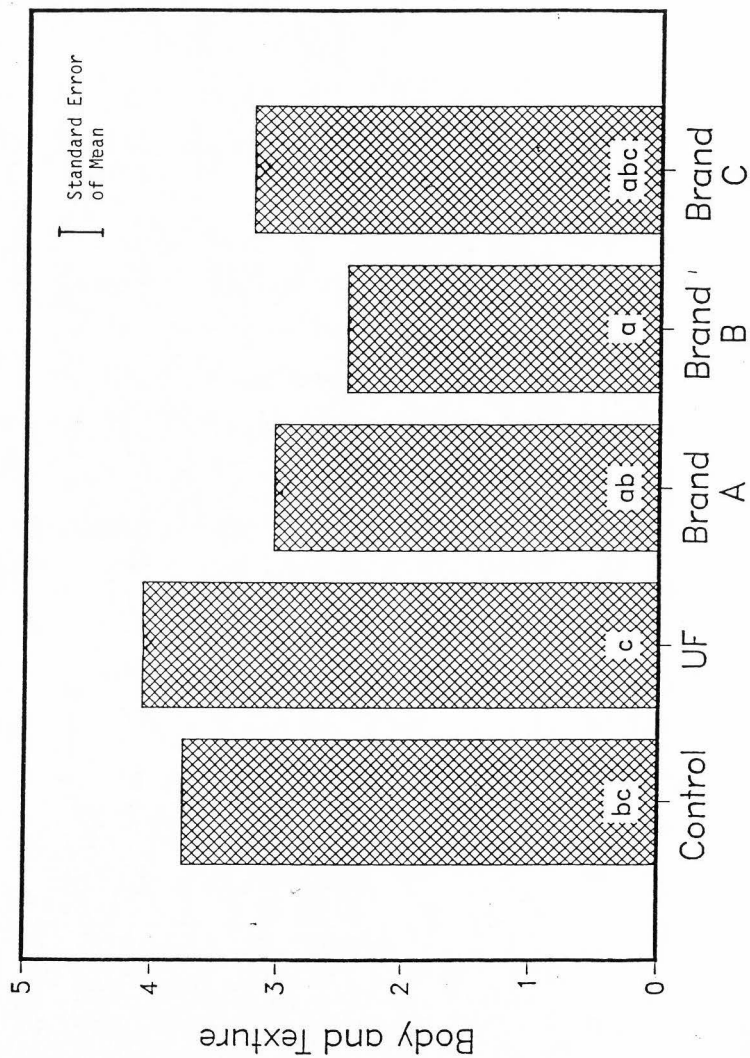


Figure 5. Means for body and texture of cottage cheese samples: control, ultrafiltered, brand A directly set, brand B directly set and brand C cultured skim milk. Bars with the same letter are not significantly different ( $p < 0.05$ ). Quality code is: 1 = poor, 2 = fair, 3 = good, 4 = very good, and 5 = excellent.





### Overall

Mean overall scores are shown in Figure 6. At  $\alpha = 0.05$ , no significant difference was observed among judges. However, the cheese and trials were significantly different from each other. No significant difference was observed between UF and control cheeses which were judged very good. Both were better than two of three commercial samples and equal to the third.

Differences observed among trials probably resulted from the use of different skim milk in each trial.

These evaluations indicate that the quality of direct acid UF cottage cheese was as good as cheese made from normal skim milk from the same lot. Control and experimental cheeses were judged as good or better than commercial cottage cheese manufactured in this area.

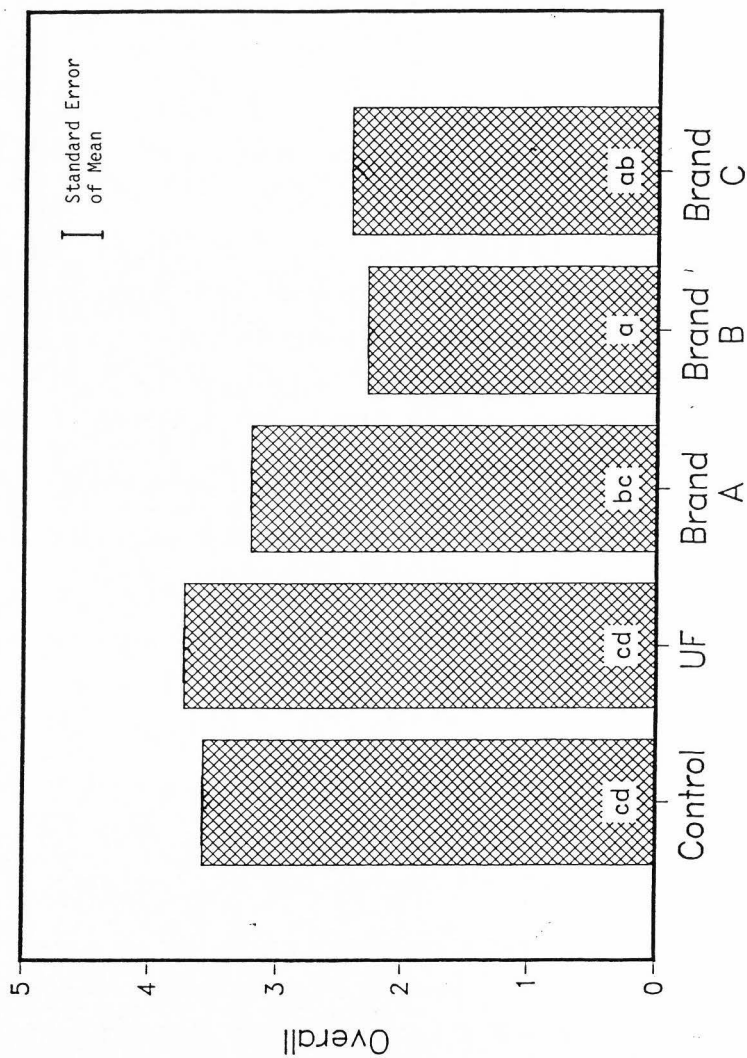
Sensory scores are summarized in Appendix 6.

### General Observations

It was difficult to produce UF cottage cheese curd with a total solids less than 22-23%. Therefore the slight yield increase that was realized by adjusting to 20% total solids probably could not be attained under plant conditions.

Excellent firmness of UF cottage cheese curd at cutting makes it a good candidate for continuous cottage cheese making by the Ernstrom (25) process. It is recommended that efforts be made to use UF skim milk in the continuous process based on complete acidification of cold retentate, then warming in a tubular heat exchanger (25).

Figure 6. Means for overall of cottage cheese samples:  
control, ultrafiltered, brand A directly set, brand B  
directly set and brand C cultured skimmilk. Bars  
with the same letter are not significantly different  
( $p < 0.05$ ). Quality code is: 1 = poor, 2 = fair,  
3 = good, 4 = very good, and 5 = excellent.



## CONCLUSIONS

1. Cottage cheese with good texture and flavor was made from UF skim milk retentate (9.1% protein)
2. The uncreamed cottage cheese made by ultrafiltered retentate was always higher than 20% total solids. When not corrected to 20% total solids, there was no increase in yield over control.
3. When corrected to 20% total solids, yields of UF curd averaged 2.24% greater than the control per 100 kg skim milk and also 2.24% greater based on yield per kilogram original milk protein.
4. Unheated retentate formed a curd that was too tough to cut. Heating to 76.5°C for 16 s improved cuttability. Heating to higher temperature resulted in weak, shattered curd.
5. The UF process resulted in increased production per vat of retentate (45% volume reduction).
6. Cottage cheese curd made from unacidified ultrafiltered retentate became transparent and gelatinous after creaming. This problem was eliminated by acidification of skim milk (pH 5.8) before ultrafiltration.

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## APPENDIXES



APPENDIX 1Cottage Cheese Score Card

Name \_\_\_\_\_ Date \_\_\_\_\_

Evaluate the following samples for the given criticisms placing a checkmark to the right of the appropriate criticisms. Enter a numerical value to the right of "score" for each category based on the following scale:

<u>Score</u>	<u>Description</u>
5	Excellent
4	Very good
3	Good
2	Fair
1	Poor

If no criticisms are checked, then a score of 5 is given.

## COTTAGE CHEESE SCORE CARD

Perfect Score	Criticism	Sample No.				
		1	2	3	4	5
Flavor	Contestant Score					
	Grade      Score Criticism					
Body and Texture	Acid					
	Bitter					
	Diacetyl					
	Feed					
	Fermented/Fruity					
	Flat					
	Foreign					
	High Salt					
	Lacks Freshness					
	Malty					
	Metallic					
	Musty					
	Oxidized					
	Rancid					
	Unclean					
Yeasty						
Lacks Fine Flavor						
Body and Texture	Contestant Score					
	Grade      Score Criticism					
Appearance & Color	Firm/Rubbery					
	Gelatinous					
	Mealy/Grainy					
	Pasty					
	Weak/Soft					
	Contestant Score					
	Grade      Score Criticism					
	Free Cream					
	Free Whey					
	Lacks Cream					
	Matted					
	Shattered Curd					
	Slimy					
	Surface discolored					
	Translucent					
	Unnatural Color					
Allowed perfect in contest	5.0	5.0	5.0	5.0	5.0	
OVERALL	Contestant score					

## APPENDIX 2

## Statistical Tables

Table 6. Analysis of variance of moisture of uncreamed cottage cheese

SV	df	SS	MS	F	Significant alpha level
Treatment	1	5.6842	5.6842	7.966	N.S.
Trial	2	1.0690	0.5345	0.7491	N.S.
Error	2	1.4270	0.7135		
Total	5				

Table 7. Analysis of variance of protein of uncreamed cottage cheese

SV	df	SS	MS	F	Significant alpha level
Treatment	1	4.9323	4.9323	21.03	0.0469
Trial	2	0.7663	0.3832	1.63	N.S.
Error	2	0.4690	0.2345		
Total	5				

Table 8. Analysis of variance of fat of uncreamed cottage cheese

SV	df	SS	MS	F	Significant alpha level
Treatment	1	0.0043	0.0043	0.2774	N.S.
Trial	2	0.1051	0.0526	3.390	N.S.
Error	2	0.031	0.0155		
Total	5				

Table 9. Analysis of variance of lactose of uncreamed cottage cheese

SV	df	SS	MS	F	Significant alpha level
Treatment	1	0.1262	0.1262	2.59	N.S.
Trial	2	0.3008	0.1504	3.09	N.S.
Error	2	0.0973	0.0487		
Total	5				

Table 10. Analysis of variance of calcium of uncreamed cottage cheese

SV	df	SS	MS	F	Significant alpha level
Treatment	1	$2.20 \times 10^{-3}$	$2.20 \times 10^{-3}$	110	0.0094
Trial	2	$1.23 \times 10^{-4}$	$6.15 \times 10^{-5}$	3.075	N.S.
Error	2	$4.0 \times 10^{-5}$	$2.0 \times 10^{-5}$		
Total	5				

Table 11. Analysis of variance of whey protein of uncreamed cottage cheese

SV	df	SS	MS	F	Significant alpha level
Treatment	1	$4.196 \times 10^{-5}$	4.1958	2.3283	N.S.
Trial	2	2.7958	1.3979	0.7757	N.S.
Error	2	3.6042	1.8021		
Total	5				

Table 12. Analysis of variance of protein of uncreamed cottage cheese (adjusted to 80% moisture)

SV	df	SS	MS	F	Significant alpha level
Treatment	1	2.6666	2.6666	16.96	N.S.
Trial	2	0.4392	0.2196	1.40	N.S.
Error	2	0.3145	0.1572		
Total	5				

Table 13. Analysis of variance of fat of uncreamed cottage cheese (adjusted to 80% moisture)

SV	df	SS	MS	F	Significant alpha level
Treatment	1	$4.7833 \times 10^{-3}$	$4.7833 \times 10^{-3}$	0.52	N.S.
Trial	2	$100.0833 \times 10^{-3}$	$50.0417 \times 10^{-3}$	5.43	N.S.
Error	2	$18.4166 \times 10^{-3}$	$9.2083 \times 10^{-3}$		
Total	5				

Table 14. Analysis of variance of lactose of uncreamed cottage cheese (adjusted to 80% moisture)

SV	df	SS	MS	F	Significant alpha level
Treatment	1	0.091233	0.0912	2.3030	N.S.
Trial	2	0.2956	0.1479	3.7348	N.S.
Error	2	0.07927	0.0396		
Total	5				

Table 15. Analysis of variance of calcium of uncreamed cottage cheese (adjusted to 80% moisture)

SV	df	SS	MS	F	Significant alpha level
Treatment	1	$1.9473 \times 10^{-3}$	$1.9473 \times 10^{-3}$	62.18	0.019
Trial	2	$0.1073 \times 10^{-3}$	$0.0537 \times 10^{-3}$	1.72	N.S.
Error	2	$0.06267 \times 10^{-3}$	$0.03134 \times 10^{-3}$		
Total	5				

Table 16. Analysis of variance of whey protein of uncreamed cottage cheese (adjusted to 80% moisture)

SV	df	SS	MS	F	Significant alpha level
Treatment	1	$2.1235 \times 10^{-4}$	$2.1235 \times 10^{-4}$	14.20	N.S.
Trial	2	$0.458 \times 10^{-4}$	$0.229 \times 10^{-4}$	1.53	N.S.
Error	2	$0.299 \times 10^{-4}$	$0.1495 \times 10^{-4}$		
Total	5				

Table 17. Analysis of variance of mean weight of uncreamed cottage cheese per 100 kg of skim milk.

Source	df	MS	F	Significant alpha level
Treatment	1	0.1734	94.75	0.0109
Trial	2	0.5955	325.41	0.0044
Error	2	0.0018		
Total	5			

Table 18. Analysis of variance of mean weight of uncreamed cottage cheese per kg of protein.

SV	df	MS	F	Significant alpha level
Treatment	1	0.2204	259.29	0.0047
Trial	2	0.0101	11.88	N.S.
Error	2	0.0009		
Total	5			

Table 19. Analysis of variance of sensory evaluation for flavor of cottage cheese samples.

SV	df	MS	F	Significant alpha-level
Treatment	4	0.7145	0.4633	N.S.
Judge	3	8.004	5.1893	0.0136
Error A	12	1.5424		
Trial	2	1.9542	5.0800	0.0139
Trial x Treatment	8	0.3396	0.8828	N.S.
Error B	30	0.3847		
Total	59			

Table 20. Analysis of variance of sensory evaluation for appearance and color of cottage cheese samples.

SV	df	MS	F	Significant alpha level
Treatment	4	4.2042	3.6960	0.0373
Judge	3	0.6444	0.5665	N.S.
Error A	12	1.1375		
Trial	2	1.6625	3.7520	0.0374
Trial x Treatment	8	0.8604	1.9418	N.S.
Error B	30	0.4431		
Total	59	0.9475		

Table 21. Analysis of variance of sensory evaluation for body and texture of cottage cheese samples.

SV	df	MS	F	Significant alpha level
Treatment	4	4.9104	3.7473	0.0358
Judge	3	4.550	3.4720	N.S.
Error A	12	1.3104		
Trial	2	1.6667	3.6367	0.0407
Trial x Treatment	8	1.1104	2.4229	0.0398
Error B	30	0.4583		
Total	59	1.2150		



Table 22. Analysis of variance of sensory evaluation for overall of cottage cheese samples.

SV	df	MS	F	Significant alpha level
Treatment	4	4.8063	4.5866	0.0196
Judge	3	1.5010	1.4324	N.S.
Error A	12	1.0479		
Trial	2	6.3542	18.1031	0.0005
Trial x Treatment	8	1.1313	3.2231	0.0094
Error B	30	0.3510		
Total	59	0.9493		

### APPENDIX 3

#### Composition of uncreamed cottage cheese

% i		Trial I		Trial II		Trial III	
		Control	UF	Control	UF	Control	UF
Moisture	AV	77.88	77.27	78.91	76.59	80.00	77.09
	SD	0.22	0.021	0.04	0.009	0.23	0.13
Protein	AV	17.83	19.32	17.44	18.79	16.40	19.00
	SD	0.25	0.25	0.25	0.24	0.23	0.22
Fat	AV	1.32	1.29	1.31	1.07	0.93	1.04
	SD	0.016	0.026	0.029	0.017	0.012	0.017
Lactose	AV	1.14	1.10	1.40	1.73	1.33	1.91
	SD	0.05	0.017	0.057	0.07	0.04	0.06
Calcium	AV	0.0525	0.0932	0.0533	0.0823	0.039	0.085
	SD	0.003	0.003	0.004	0.0002	0.0002	0.0002
Whey Protein	AV	0.5767	0.6627	0.6176	0.6776	0.5321	0.6658
	SD	0.02	0.059	0.02	0.021	0.01	0.029

AV = Average

SD = Standard Deviation

Appendix 4  
Composition of cottage cheese

		<u>Trial I</u>		<u>Trial II</u>		<u>Trial III</u>	
<u>%</u>		<u>Control</u>	<u>UF</u>	<u>Control</u>	<u>UF</u>	<u>Control</u>	<u>UF</u>
Moisture	AV	76.24	77.27	78.70	76.59	78.83	77.09
	SD	0.05	0.02	0.16	0.009	0.05	0.13
Protein	AV	12.70	13.18	12.78	14.04	12.59	14.06
	SD	0.009	0.02	0.06	0.03	0.03	0.08
Fat	AV	5.93	6.57	5.84	4.62	4.26	4.21
	SD	0.12	0.03	0.05	0.11	0.13	0.13
Lactose	AV	1.63	1.81	2.40	2.56	2.28	2.50
	SD	0.13	0.009	0.13	0.13	0.05	0.11
Calcium	AV	0.075	0.106	0.070	0.090	0.061	0.089
	SD	0.0003	0.0002	0.0004	0.002	0.0004	0.002

AV = Average

SD = Standard Deviation

## Appendix 5

### Composition of uncreamed cottage cheese (adjusted to 80% moisture)

%	Trial I		Trial II		Trial III	
	<u>Control</u>	<u>UF</u>	<u>Control</u>	<u>UF</u>	<u>Control</u>	<u>UF</u>
Moisture	80.00	80.00	80.00	80.00	80.00	80.00
Protein	17.36	18.16	17.20	17.99	16.40	18.31
Fat	1.29	1.25	1.29	1.02	0.93	1.00
Lactose	1.11	1.06	1.38	1.66	1.33	1.84
Calcium	0.052	0.090	0.052	0.079	0.039	0.082
Whey protein	0.521	0.583	0.586	0.619	0.532	0.581

# APPENDIX 6

## Mean sensory scores for cottage cheese samples

<u>Samples</u>	<u>Flavor</u>	<u>Appearance &amp; Color</u>	<u>Body &amp; Texture</u>	<u>Overall</u>
Control	3.63 <sup>a</sup> ± 0.062	3.38 <sup>abc</sup> ± 0.056	3.75 <sup>bc</sup> ± 0.015	3.58 <sup>c</sup> ± 0.032
UF	3.50 <sup>a</sup> ± 0.077	3.80 <sup>c</sup> ± 0.023	4.08 <sup>c</sup> ± 0.023	3.73 <sup>c</sup> ± 0.044
Commercial A	3.46 <sup>a</sup> ± 0.062	3.29 <sup>abc</sup> ± 0.062	3.04 <sup>ab</sup> ± 0.062	3.21 <sup>bc</sup> ± 0.058
Commercial B	3.04 <sup>a</sup> ± 0.032	2.63 <sup>a</sup> ± 0.015	2.46 <sup>a</sup> ± 0.024	2.29 <sup>a</sup> ± 0.023
Commercial C	2.75 <sup>a</sup> ± 0.053	2.79 <sup>ab</sup> ± 0.112	3.21 <sup>abc</sup> ± 0.109	2.42 <sup>ab</sup> ± 0.099
LSD	0.990	0.853	0.916	0.819

Means in the same column with the same letter are not significantly different ( $p < 0.05$ ). Each mean is the average of 12 responses.

Product was rated on a grading scale of 1 = poor, 2 = fair, 3 = good, 4 = very good and 5 = excellent

PROJECT TITLE: Cogeneration of Biogas and Single Cell Protein From Ultrafiltration Permeate and Whey

11. Project Details

Objectives:

Preliminary studies we have completed indicate that the UASB digester has great potential to benefit the dairy industry through , co-production of biological protein and biogas and pretreatment of liquid wastes. However, we have not yet determined optimal process parameters or how the process will work on ultrafiltration permeate from milk or whey. We would expect from our related work that permeate will require shorter hydraulic retention times than whole whey. We do not yet know the yield or quality of biogas or biological protein that can be produced from permeate. Nor do we know the maximum efficiency of pollutant (e.g. biochemical oxygen demand (BOD) and chemical oxygen demand (COD), TS, nitrogen and phosphorus) removal. Therefore, we propose to:

1. Digest whole whey and whey milk permeate in a novel, computer controlled, anaerobic digester that coproduces biological protein and biogas.
2. Investigate the effect of supplemental micronutrient and macronutrient addition on overall reactor stability and performance.
3. Investigate quantity and quality of biological protein and methane that can be produced by determining mass balance relationships (kinetics) between organics removal, methane production and biological protein production.

PROJECT TITLE: Cogeneration of Biogas and Single Cell Protein From Ultrafiltration Permeate and Whey

Background Information: (continued)

Ross (1984) also conducted a compositional analysis of the sludge pellets and found them to contain 90% VSS, with some 66% of their total mass represented as crude protein, and 11 to 12.5% being nitrogen. The high protein content of this material makes it attractive for use as a potential protein source for livestock. Hansen (1985) conducted preliminary investigations related to the potential of this UASB pellet material as a feed substitute for young male and female mice. A control group was fed commercial mouse feed, while a treatment group was fed commercial mouse feed with an amendment of 10% UASB pellet single cell protein (SCP) material over a two month period. At the end of the study period, no statistically significant clinical behavioral nor mouse body weight differences were observed between the two groups, suggesting the feasibility of the use of such SCP as a safe and cost effective feed amendment and protein source.

Summary. The upflow anaerobic sludge blanket process has been actively researched since its conception in the late 1970's. Much of the research has involved treatment of carbohydrate type wastes. Data for whey/permeate is limited however. A considerable amount of work has been done with UASB digestion of dairy plant waste which has a composition significantly different than cheese whey/permeate. There are no known data on the value or quantity of biological protein that can be harvested from a UASB digester. It has been well established that anaerobic biological protein has a significantly different amino acid composition than aerobically generated solids and it is usually ~~more valuable as an animal feed supplement.~~ Results of our previous

PROJECT TITLE: Cogeneration of Biogas and Single Cell Protein From Ultrafiltration Permeate and Whey

Summary: (continued)

work with UASB treatment of a meat processing waste suggest that the biological protein produced by the UASB has significant value as an animal feed supplement.

It appears that the UASB reactor represents a feasible and viable alternative for the anaerobic treatment of high COD/lactose whey and permeate wastes that provides the benefits of high solids retention time fixed-film systems. These benefits include high process loading rates and process stability. The UASB avoids the limitations associated with fixed-film systems, ie., high media costs, use of reactor volume by reactor media, potential reactor clogging characteristics, and lack of reactor SRT control. UASB sludge pellet formation data, although limited, also indicate that carbohydrate wastes, such as whey permeate, are particularly well suited for sludge blanket formation. Because the UASB reactor provides a mechanism for sludge bed SRT control, it allows for both COD removal/waste treatment optimization and maximization of sludge production for recovery of valuable SCP, and consequently, optimal total product recovery. The UASB represents a highly promising process for the effective treatment of whole whey and whey permeate that can represent an efficient and cost-effective treatment option once design limitations and process operating characteristics are classified for this waste stream.



PROJECT TITLE: Cogeneration of Biogas and Single Cell Protein From Ultrafiltration Permeate and Whey

Design of Proposed Project:

Apparatus:

An improved UASB reactor configuration for the optimal treatment of whey/permeate has been developed in the Nutrition and Food Sciences, Department at Utah State University. Features of the reactor include computer control of loading schedule, pH, and temperature. Values of pH, temperature, gas production and loading rate will be automatically acquired and recorded. The computer monitors and controls pH via a New Brunswick model pH-40 controller. Gas production is monitored with a unique device developed in our laboratory (Figure 2). The computer will control loading based on a given set point and loading schedule unless there is a sharp or rapid drop in pH which triggers an alarm and stops the loading operation.

A Leading Edge model D microcomputer with Metrabyte DASCON 1 interface is used for data acquisition and process control. Loading rate is controlled with a computer controlled feed pump. Temperature is controlled with a mercury thermoregulator. The computer will keep records of temperature, pH, loading rate, loading schedule and gas production.

Analyses:

Reactor performance will be evaluated based on COD and BOD removal, volatile acids concentration, nutrient (N, P and Ca) removal, and gas and SCP production and quality. Gas quality is based on methane percentage. SCP quality is based on amino-acid composition. All analyses will be conducted in accordance with standard techniques (Standard Methods, APHE, AWWA, WPCF 1975; and B. Hingmeyer, et al.,

1984). Equipment and facilities available for all analyses are detailed under Available Facilities and Equipment.

#### Methodology:

##### Whey and Permeate Digestion Experimental Design

Research pertaining to the first objective will evaluate the effects of five major variables on the performance of our UASB system. These variables are 1) the loading schedule, i.e. continuous vs. slug loading of the reactor, 2) the loading rate (mass of feed per unit reactor volume per day), 3) the hydraulic residence time, 4) the solids retention time (a surrogate variable used to describe the mean cell residence time of the active biomass in the system), and 5) the reaction temperature.

A considerable acclimation time is required to evaluate the effect of each variable. Usually a period of three to four times the solids residence time (SRT) is required to ensure that the reactor has stabilized under the new set of experimental conditions. As typical SRTs are on the order of 5 to 15 days, anywhere from 15 to 60 days are required to complete one run. Since the number of variables of interest is quite large, even if only two levels of each variable are studied, from 150 to 600 days would be required for the first phase of the testing using duplicate reactors.

Factorial experimental designs are efficient for finding the magnitude of the linear effects of several variables and the interactions between those variables (Box, et al., 1978). To visualize this design, consider the study of 2 variables, say  $X_1$  and  $X_2$  at two levels each. The magnitudes of the variables are scaled so that they can be coded as -1 for a low level and +1 for a high level. Using the

factorial design, experiments are run at all combinations of levels of the variables. For  $n = 2$  variables at two levels, the combinations are  $(X_1, X_2) = (-1, -1), (1, -1), (-1, 1),$  and  $(1, 1)$ . In general, the number of experiments required for  $n$  variables at  $m$  levels is  $m^n$ . Thus for  $n = 2$  and  $m = 2$  the number of runs is  $2^2 = 4$ . A model for the effects of the variables and the interactions between the variables' is

$$y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + e \quad (1)$$

where  $y$  is the value of the dependent variable (eg. gas production rate, single cell protein production rate, etc.),  $X_1$  and  $X_2$  are the levels of the independent variables (eg. temperature, loading rate, etc.),  $b_1$  and  $b_2$  are the coefficients for the effects of the variables, estimated from the data,  $b_{12}$  is the coefficient for the interaction between the main variables,  $b_0$  is the value of  $y$  when  $X_1 = X_2 = 0$ , and  $e$  is the error. The coefficients  $b_1$  and  $b_2$  estimate the change in the dependent variable when the values of the independent variables are changed from 0 to +1. The term "main effects" is used to estimate the effect of changing the levels of the independent variables from -1 to 1, so that the values of  $b_1$  and  $b_2$  are equal to 1/2 the values of the main effects.

When many variables are to be studied at once, the number of runs can be reduced by associating some of the main effects with high order interactions between variables. This type of design is known as a fractional factorial design. For the experiments proposed here,  $n = 5$ , so that for a full factorial design at two levels ( $m = 2$ ), the number of runs is  $2^5 = 32$ . If two of the variables can be associated with high order interactions, then the number of runs is  $2^{5-2} = 8$ . In fact, the highest number of variables that can be studied in  $k$  runs, assuming that all interactions are negligible, is  $k-1$ , making the fractional factorial

experimental design very efficient for selecting a subset of important variables from a large number of candidates.

The design chosen for this study is a  $2^{5-2}$  fractional factorial design and is described in Table I. This 8 run design allows the independent estimation of the main effects of loading schedule, loading rate, and HRT while the main effects of SRT and temperature are confounded with the three-way interaction between loading schedule, loading rate, and HRT, and the two way interaction between loading rate and HRT, respectively. The model to be used to estimate the main effects is

$$y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4^*X_4 + b_5^*X_5 + e$$

\* these effects are confounded with interactions

Each run will be randomized, that is the eight runs will be done in random order so that the statistical significance tests are not compromised.

Further time savings will be realized by providing more than one experimental apparatus. A total of four units each similar to the one shown in Fig. 1, will be operated in parallel; two will be run as duplicates to assess the expected variability between duplicate reactors, while the remaining two will be operated as set out in Table 1. The two duplicate reactors will be randomly assigned among the four available during each time period so that an unbiased estimate of the between reactor variance can be obtained. It is expected that the between reactor variance will be the same for all experimental conditions, and can be used when estimating the statistical significance of the main effects and interactions.

The runs set out in this design will require 4 SRTs to stabilize before data collection for comparison of performance can begin. Three experimental periods will be needed to carry out all runs since, with four reactors using one duplicate, three runs can be done during one period. The system will be operated for 4 times the longest SRT in each group of 15 days or 60 days. Assuming one week of maintenance between time periods, about 200 days, or 6.5 months, is required for completion of this phase of the research. At the end of this period, estimates of the main effects will be calculated and variables will either be retained or eliminated for the next set of runs.

This experiment demands frequent (several times weekly) wastewater analyses including: influent, effluent and reactor COD, solids (TS, SS, VSS) and volatile acids. Therefore, a technician will be needed to perform the necessary laboratory work and calculations.

#### Micronutrient Addition Experimental Design

##### Rationale

It has become apparent from recent advances in the application of anaerobic systems to industrial waste treatment that due to the unique nature of nutritional requirements of the methane producing organisms, consideration of only the traditional nutrient levels, i.e., nitrogen and phosphorous, is not sufficient for an adequate assessment of the true anaerobic treatability of these wastes (Speece 1983). Such trace elements as iron, cobalt, nickel and sulfide have been shown to be of particular importance to the growth and reproduction of the methanogens, and consequently to the ultimate success of waste stabilization utilizing anaerobic processes (Speece and McCarty, 1964; Hoban and Van den Berg, 1979; Owen, et al., 1979; Diekert, et al., 1981; Scherer and

Sahm, 1981). Because of the growing realization of the importance of these trace minerals to the performance of anaerobic waste treatment, a preliminary degradation in the UASB reactors will be conducted using batch bioassay procedures with UASB sludge that has been developed in preliminary studies.

#### Experimental Design

The micronutrient addition experiments will be conducted over a four week period during which time gas volume and composition measurements will be made according to the following schedule:

Week 1: Volume and composition measurements made daily

Week 2: Volume and composition measurements made every 2 days

Weeks 3 & 4: Volume and composition measurements made every 3 days

Triplicate reactors will be used for each treatment condition employed in the factorial design presented in Table 2. As indicated in Table 2, a total of  $16 \times 3 = 48$  reactors will be required for evaluation of the effect of iron, cobalt/molybdenum, nickel and sulfur additions on the treatability of whey permeate waste using UASB reactor sludge. This experimental design will allow the identification of optimal micronutrient addition treatment(s), either as individual elements or as components of a complex nutrient mixture, that will be utilized in full scale sludge bed treatment studies during subsequent phases of the project.

#### Experimental Procedures:

Treatability assays will be conducted according to procedures described by Owens, et al. (1981) utilizing 250 ml glass serum bottles. The procedures entail the preparation of various sample/defined media solutions which are anaerobically incubated for 30 days at 25 to 35°C,

during which time gas volume and methane composition data are monitored. Anaerobic treatability is expressed as ml methane/g COD in the sample for each waste assayed, with comparison made with respect to an unfed control sample. Treatability assays conducted in this study will be modified slightly to include: 1) unamended\* UASB sludge samples as an unfed control, 2) unamended UASB sludge fed whey permeate waste to 2 g COD/l as a fed control, and 3) micronutrient amended UASB sludge combinations (as per Table 2) as the treatment samples. Levels of micronutrient amendment to be used are based on recommended literature values for methanogen nutrient requirements. Micronutrients are to be added to the raw whey permeate waste to produce the following solution concentrations:

Iron = 345 mg/g COD as $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$	(Owen et al. 1979)
Cobalt = 28 mg/g COD as $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	(Owen et al. 1979)
Molybdenum = 2.4 mg/g COD as $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	(Owen et al. 1979)
Nickel = 0.7 mg/g COD as $\text{NiCl}_2$	(Dickert et al. 1981)
Sulfur = 233 mg/g COD as $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$	(Owen et al. 1979)

Gas volume and composition measurements will be made using gas-tight glass syringes (Owens et al. 1981), with gas composition determined using gas chromatographic procedures for the separation and quantification of primary components of the product gas, i.e., methane, carbon dioxide, oxygen, nitrogen and hydrogen sulfide. Gas volume and composition data collected over the study period according to the schedule presented above will be analyzed within the factorial design

\*The term unamended pertains to micronutrients. Macronutrients (NP & K) will be added when necessary to insure sufficiency for the microorganisms.

described earlier to identify the optimal treatment(s) to be applied in full scale experimentation. Optimal treatment(s) will be identified based on maximum methane production rate and methane per gram COD removed results observed during incubation.

#### Time Schedule

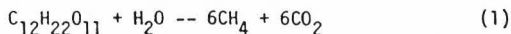
The following time schedule is proposed for the completion of the micronutrient addition treatability studies. This schedule includes the requisite comprehensive waste evaluation, data collection and data evaluation periods necessary for successful determination of optimal treatment options.



## Biological Protein and Methane Production

One of the advantages of the UASB system is that excess cell mass (biological protein) can be easily removed. Periodic "harvesting" of biological protein, if done in a proper manner, will help insure well performing reactors.

during this part of the research reactors will be operated in an optimal fashion based on data collected for objectives one and two. Optimum performance is when the reactors are removing the greatest amount of BOD (or COD) per unit time while producing an acceptably clean effluent. The amount of biological protein that can be removed without significantly lowering the efficiency of the reactors can be estimated using COD balances. COD removed from substrate is converted either into biogas (methane, carbon dioxide) or new cells. Lactose is the constituent in whey and permeate that contributes most of the COD and is most readily removed with the short hydraulic retention times in UASB reactor. The stoichiometry of lactose conversion to biogas is:



For a 4.45% lactose solution (similar to composition of whey):

$$\begin{aligned} & \frac{44.5 \text{ g lactose}}{1 \text{ l solution}} \times \frac{1 \text{ mole lactose}}{342 \text{ g}} \rightarrow 6 \times \frac{44.5}{342} \times \frac{22.4 \text{ l } CH_4}{\text{mole } CH_4} \\ & = \underline{17.48 \text{ l } CH_4} \text{ (at standard temperature and pressure) per 1 l of} \\ & \quad \text{lactose solution} \end{aligned}$$

Theoretical COD value of 1 l of lactose solution:

$$\begin{aligned} & C_{12}H_{22}O_{11} \times 12O_2 \rightarrow 12CO_2 + 11H_2O \quad (2) \\ & 12 \text{ mole } O_2 \times \frac{32 \text{ g}}{\text{mole } O_2} \times \frac{1 \text{ mole lactose}}{342 \text{ g}} = 1.1228 \text{ g} \cdot \text{g}^{-1} \text{ lactose} \\ & 1.1228 \times \frac{44.5 \text{ g lactose}}{1 \text{ l lactose solution}} = \underline{49.96 \text{ g COD per 1 lactose solution}} \end{aligned}$$

Phase 1: Waste Evaluation	1 Month
Phase 2: Treatability Assays	1 Month
Phase 3: Data Reduction and Evaluation	<u>1 Month</u>
TOTAL	3 Months

Table 2. Factorial design table for micronutrient addition experiments

Runs	Fe	Co/Mo	NH	S
1	-1	-1	-1	-1
2	1	-1	-1	-1
3	-1	1	-1	-1
4	1	1	-1	-1
5	-1	-1	1	-1
6	1	-1	1	-1
7	-1	1	1	-1
8	1	1	1	-1
9	-1	-1	-1	1
10	1	-1	-1	1
11	-1	1	-1	1
12	1	1	-1	1
13	-1	-1	1	1
14	1	-1	1	1
15	-1	1	1	1
16	1	1	1	1

---

-1 indicates LACK OF ADDITION of following levels of micronutrients  
 1 indicates ADDITION of following levels of micronutrients

Fe = 345 mg/g COD as  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$   
 Co/Mo = 28 mg/g COD as  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  and  
 2.4 mg/g COD as  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$   
 Ni = 0.7 mg/g COD as  $\text{NiCl}_2$   
 S = 233 mg/g COD as  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$

Predicted biogas yield:

$$\frac{12.48}{49.96} \approx \frac{0.35 \text{ l CH}_4}{\text{g}} \text{ per g of COD added}$$

The mass of cells (biological protein) that can be produced per g of lactose can be illustrated with a carbon balance. The general formula for cell mass is  $C_5H_7NO_2$ , therefore 1 mole lactose can supply carbon to produce 2.4 mole of cell mass or 271 g cell mass per 342 g of lactose. This is also equivalent to  $0.79 \text{ g cell mass} \cdot \text{g}^{-1} \text{ lactose}$ . The amount of cell mass that can be produced on the basis of COD removed is found by dividing the cell yield into the theoretical COD value for 1 g of lactose or  $1.1228 \text{ g} \cdot \text{g}^{-1} \text{ lactose}$ :

$$\frac{1.1228 \text{ g} \cdot \text{g}^{-1} \text{ lactose}}{0.79 \text{ g} \cdot \text{g}^{-1} \text{ lactose}} = \frac{1.42 \text{ g COD} \cdot \text{g}^{-1} \text{ cell mass}}{\text{g}} \quad (3)$$

Knowing that 350 ml of methane can be produced or 0.7 g of cell mass can be produced per g of theoretical COD removed, we can establish mass balance relationships between organics removal (COD removal), methane production and biological protein production. We recognize that there are components in whey other than lactose that may have a significant contribution to methane production and cell synthesis. However, the simplified model is a good starting point. A more complex model will be developed if necessary.

COD removal will be determined with standard analysis (APHA, 1975) performed at least bi-weekly. BOD's will also be run periodically to establish a BOD/COD ratio. Gas production will be measured by acidified water displacement in a container of known volume. The methane percentage in the gas will be measured using gas chromatography.

Biological protein will be harvested from the effluent by sedimentation if it will settle or by centrifugation. This material will be dried, weighed, and analyzed to establish an amino acid profile.

In addition, biological protein may be harvested from the sludge bed of the reactors.

Three reactors will be used for this aspect of the work each loaded at the same optional rate. Reactor one will be kept as a control, no harvesting of cell mass; reactor two will be overharvested--cell mass extracted from the effluent and taken from the sludge bed is slightly greater than the theoretical production of cell mass - until efficiency begins to drop. Reactor three will be harvested at the theoretical production rate. Solids analyses (total, suspended and volatile suspended) will be run on a regular basis along with the COD analyses.

Harvested biological protein will be analyzed weekly for protein content by the Kjeldahl method. At least three amino acid analyses using HPLC (precolum derivatization with phenylisothiocyanate) will be run on composite samples to determine protein quality. Samples will be dried at 100°C and frozen to enable storage until a sufficient amount of composite sample has been collected for amino acid analyses. Amino acid analyses will be performed in our laboratories by a technician and graduate student under the direction of the principal investigator. The experimental portion of this work will be accomplished in 6 months and an additional month will be needed for data reduction.

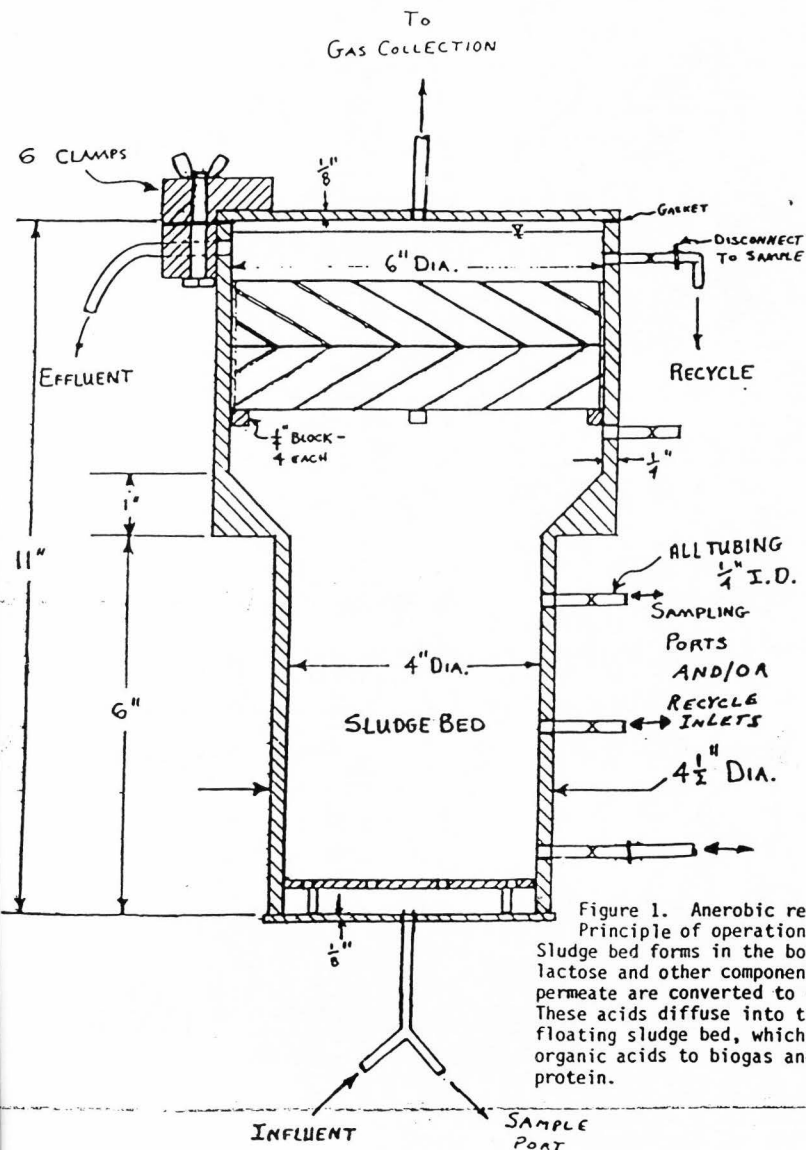


Figure 1. Anaerobic reactor  
Principle of operation:

Sludge bed forms in the bottom where lactose and other components of whey/permeate are converted to organic acids. These acids diffuse into the upper, floating sludge bed, which converts organic acids to biogas and biological protein.

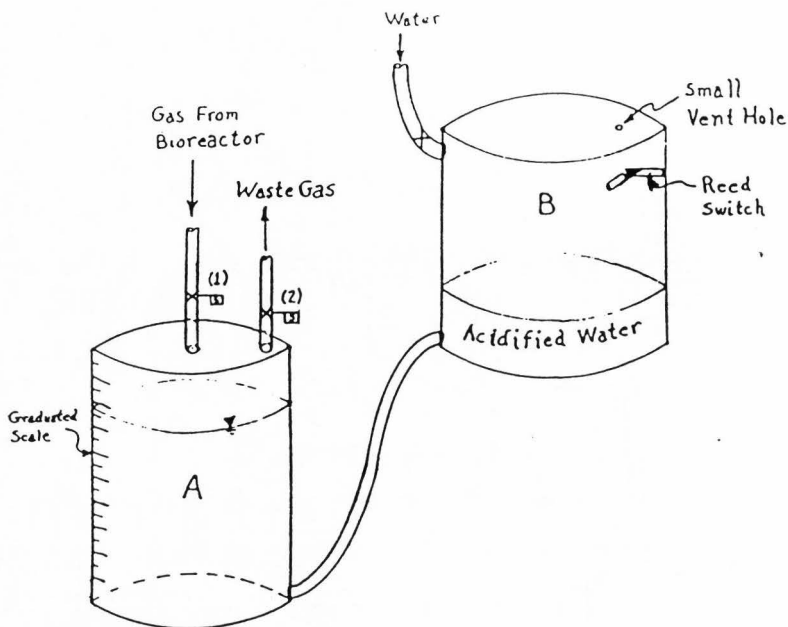


Figure 2. Biogas collection and volume measurement system.

Principle of operation: Biogas forces water to container B from A. Water level is detected by reed switch which temporarily opens solenoid valve 2 and closes valve 1 until water returns to A. The reed switch also signals the computer, which logs the time of the event.

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# Time Table for Completion of Project

	1	3	6	9	12	15	18
Build two additional reactors and interface to computer	<hr/>						
Whey and permeate digestion experiments		<hr/>					
Micronutrient addition experiments		<hr/>					
Biological protein and methane production experiments					<hr/>		
Report preparation						<hr/>	

PROJECT TITLE: Cogeneration of Biogas and Single Cell Protein From Ultrafiltration Permeate and Whey

Available Facilities and Equipment:

Food engineering laboratories contain over 3000 ft<sup>2</sup>. All utilities are available including steam and compressed air. A large amount of freezer and refrigerated space is available. Fermentations can be carried out in a temperature controlled room.

The Water Research Laboratory Environmental Quality Lab is 11,000 ft<sup>2</sup> and includes 1) a microbiology complex for bacterial, algae and viral research; 2) four constant temperature bioassay and research labs; and 3) a 4800 ft<sup>2</sup> wet chemistry laboratory.

Media. Whey is available from the university dairy processing facility which is part of the Dept. of Nutrition and Food Sciences. The facility supplies dairy products to the campus and local community. Whey or milk permeate can be made from equipment available in departmental laboratories or it can be obtained from cheese processors within 5 miles of campus.

Bench Scale Waste Treatment Facilities. Complete facilities are available for bench and pilot scale industrial waste treatment. A 1500 ft<sup>2</sup> welding, electrical and carpentry shop is available for fabricating bench scale systems. A staff of these skilled physical shop staff can construct laboratory scale fermentors and other equipment designed by the personnel on the project.

Control Equipment - pH and Temperature. Automatic pH and temperature control apparatus are available. The food engineering laboratories are emphasizing computer based process control and data acquisition. Computers and interfacing equipment are being acquired as rapidly as possible with appropriated (state) funds. Once these

PROJECT TITLE: Cogeneration of Biogas and Single Cell Protein From Ultrafiltration Permeate and Whey

Available Facilities and Equipment: (continued)

computer based systems are available, pH and temperature control apparatus will be interfaced to the computer for added versatility and more efficient handling of data.

Analytical Capabilities. Major Ion and Nutrient Analyses can be performed using a Technicon Autoanalyzer II dual channel automated analytical system with dual channel digital printer. Titrimetric (indicators and pH meters available), colorimetric, and potentiometric millivolt methods are also available. Balances, differential fraction collectors, centrifuges, filtration apparatus as well as all common laboratory glassware (flasks, beakers, bottles, burettes, pipettes, etc.) are available.

Trace Metal Analyses can be performed with an atomic absorption spectrophotometer (Perkin-Elmer Zeeman/5000 System). Present capability exists for Al, Ag, As, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, Pb, Se, and Zn using either flame or flameless techniques.

A rapid sequential inductively coupled plasma emission spectrometer has been acquired through funds provided by the National Science Foundation Equipment Grant Program. This instrument can analyze for the same elements as the atomic absorption spectrophotometer, as well as phosphorus, silicon, and sulfur. Analyses can be performed at rates in excess of 15 elements per minute using a demountable torch and corrosion-resistant spray chamber with a corrosion-resistant cross flow nebulizer. Typical analytical precision is better than 3 percent relative at this high speed. If better precision is required, i.e., less than 1 percent relative, a slower speed may be selected. Because

PROJECT TITLE: Cogeneration of Biogas and Single Cell Protein From Ultrafiltration Permeate and Whey

Available Facilities and Equipment: (continued)

it is a sequential system, analyses are performed at the optimum analytical wavelength for each element being determined and each matrix being analyzed. Unique software permits the analyst to develop , optimized procedures for the analysis being performed and to store these methods on floppy disk for recall and use at a later time. Data may be transferred to a mainframe computer for manipulation at a later time or transferred to a different laboratory for comparative work.

Organic Analyses including biogas can be obtained by using individual analyses with gas chromatography (thermal conductivity, flame ionization, electron capture, and nitrogen-phosphorus detectors) using programmed temperature and flow control. The nitrogen-phosphorus detector is a variation on flame ionization, and is selective and extremely sensitive to compounds containing only nitrogen and phosphorus. Two new Hewlett Packard 5880 gas chromatographs provide quality organic analysis. These gas chromatographs are equipped with BASIC programming capabilities (23K bytes), and one is equipped with a Hewlett Packard 7672A automatic injector capable of consecutive injection of 99 samples. A Gas Chromatograph/Mass Spectrometer/Data System (Hewlett-Packard 5985) is capable of both chemical ionization and electron impact mass spectrometric analysis of chemical residues using a quadrupole analyzer (mass spectral range from 10 to 1000 amu). The instrument has multiple capabilities of sample introduction to the dual switchable EI/CI ionization source (gas inlet probe, direct introduction probe and gas chromatographic inlets for both direct and jet separation). The gas chromatograph is fully temperature programmable

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Available Facilities and Equipment: (continued)

with an oven capable of housing packed columns as well as capillary columns. It is also equipped with a single FID detector. The data system (32K word memory) can perform data acquisition, background, spectra subtraction, tabulation and library searching for both qualitative and quantitative analysis. The unit is also equipped with hard copy capabilities for both the gas chromatograph and the mass spectral data.

Thin layer and column chromatography equipment are also available. A Perkin-Elmer Series 4 high performance liquid chromatograph equipped with autosampler, quaternary solvent delivery, programmable wavelength UV and fluorescence detector, and normal and reverse phase gradient elution capabilities is also available at the UWRL for evaluation of complex organic mixtures in liquid, air or soil samples.

Organic nitrogen analyses are performed using persulfate oxidation or as total nitrogen (Coleman Nitrogen Analyzer). BOD, COD, and TOC (Oceanographic International Carbon Analyzer) are also routinely analyzed.

Other equipment for characterizing the organic complement include: a Perkin-Elmer 599 infrared spectrophotometer, a JEOL FX-90-Q nuclear magnetic resonance spectrometer, a Cary 219 UV-Vis spectrophotometer, and Varian XL-300 superconducting FT nuclear magnetic resonance spectrometers, available in the Chemistry Department.

Biological Analyses can be done macroscopically or microscopically. Microbiological analyses (both enumeration and activity measurements) can be performed in the microbiological laboratory. In addition,



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Available Facilities and Equipment: (continued)

various biochemical entities can be measured in vivo and in vitro (Chlorophyll a, Turner fluorometer; phytoplankton size distribution and total volume, Coulter Counter; Warburg Analysis). An integrating photometer (SAI Technology Co., Model 3000) is available for measuring extremely low concentrations of adenosine triphosphate (ATP) for determination of microbial biomass. Light intensity of the ATP bioluminescent reaction is quantitatively measured.

Library Facilities. Utah State University has an excellent technical library containing approximately 200,000 volumes with emphasis in journals in the fields of engineering, food science, environmental sciences, toxicology, and chemistry.

Computer Facilities. Time-sharing access to four mainframe computers; two DEC VAX 11/780's, an IBM 4341, and a UNIVAC 1108. Access to the VAX computers is available through hardwired CRT terminals. Access to the latter two computers is by telephone modem. The mainframe computers are equipped with many popular canned routines such as IMSL, SPSSX, SAS, MINITAB, and REDEQL, as well as many locally produced programs. Remote line printers, a Tektronix 4006-1 graphics terminal and integrative digital XY plotter, electronic digitizers, several laser printers and a large number of DEC, IBM compatible and Macintosh Microcomputers are also available at U.S.U. and the UWRL.

## Significance of the Project to the Dairy Industry

Whey and permeate are materials of potential value that are often disposed of at considerable expense to cheese processors, especially smaller processors. Industrial dischargers of highly polluted wastewaters are required under U.S. law to develop an approved pretreatment program. The law also requires municipalities to recover revenue from industrial waste dischargers to pay for public wastewater treatment. As a result, stiff surcharges are levied on wastewater received from cheese plants that dispose of whey/permeate in the sewer and do not pretreat. In some cases, municipalities will not accept effluent from these processors regardless of surcharges unless waste is first pretreated.

The proposed research will develop a technology which can utilize whey and permeate providing economic benefit to the cheese industry because of reduced sewer fees and sale of by-products, biogas, and biological protein. This will result in lower consumer cost for dairy products and a resulting increase in consumption. An additional alternative use for whey and permeate will help prevent wastage of these by-products.

Preliminary experiments in our laboratories in digesting whey to produce SCP and biogas are promising, but much more work needs to be done. The efficiency and economics of our process for treating permeate can be estimated from results of experiments using the UASB with other food processing products.

The UASB digester will remove up to 90% of the BOD/COD and most of the odor from wastewater while producing 200 l of biogas (75-80% methane) per kg of organic matter destroyed. The UASB coproduces SCP-approximately 26 gm of a 25% high quality protein material per kg of organic matter destroyed. Its performance on permeate and whey is unknown. The value of pollutant reduction, thus avoiding sewer surcharges and meeting plant effluent limitations usually far outweighs the value of byproducts produced. When food processing wastewater including whey must be disposed of in a municipal sewer, research has shown that it is generally much cheaper to pretreat the wastewater on-site before dumping to the sewer to avoid high surcharges. The UASB is capable of performing this pretreatment in a highly economical and efficient manner.

**Project Title:** Cogeneration of biogas and single cell protein from ultrafiltration permeate and whey.

**Personnel:** C. L. Hansen, Associate Professor, Nutrition & Food Sciences, Utah State University

G. S. Choudhury, Research Associate, Nutrition and Food Sciences, Utah State University

**Funding Sources:** Western Dairy Foods Research Center  
Utah Agricultural Experiment Station

### Objectives:

1. Digest whole whey and whey milk permeate in a novel, computer controlled, anaerobic digester that coproduces biological protein and biogas.
2. Investigate the effect of supplemental micronutrient and macronutrient addition on overall reactor stability and performance.
3. Investigate quantity and quality of biological protein and methane that can be produced by determining mass balance relationships (kinetics) between organics removal, methane and biological protein production.

### Results:

In about the past 10 years, anaerobic digester design has changed radically from previous complete mix designs to digesters which retain the slow growing anaerobic bacteria. Complete mix reactors require hydraulic retention times (HRT) of 10-30 days. The new designs pass waste water through the retained sludge where it is treated within 4 h to 5 d. Organic matter in the waste is taken up by the bacteria in the sludge and converted to methane, a small amount of carbon dioxide and more biological sludge (new cells). Waste water treatment in these new "cell retention" digesters is at least as good if not better than the old complete mix designs.

We selected the upflow anaerobic sludge blanket (UASB) reactor, a sludge retention design, for our research mainly because of the ease of harvesting biological sludge. The biological sludge is a by-product of anaerobic treatment of whey permeate which can be sold as cattle or human food. Another by-product is biogas, which can be burned like natural gas for energy.

The UASB features a sludge bed which collects in the bottom half to two thirds of the reactor by gravity. Whey permeate is fed into the bottom of the reactor and passes through the sludge bed at a slow enough velocity that the sludge bed is not displaced.

#### Whey permeate digestion studies

It was more difficult than we expected to digest whey permeate in a UASB; our initial attempts were unsuccessful. We had to first complete the work of objective two to understand what the nutrient requirement were. We then redesigned our reactor with a greater length to facilitate better sludge retention. The present reactor is 81 cm (32") in length and 10 cm (4") in diameter. The top 13 cm (5") is expanded 50% to reduce velocity of treated water leaving the UASB thereby helping the bacteria to settle into the sludge bed.

Whey permeate was anaerobically digested in a USAB digester in separate experiments with different sub-objectives. The first sub-objective was to determine an upper limit on organic (COD) loading rate. The second was to determine an optimal removal efficiency. The upper loading limit was 15 mg COD/L-d with about 55-65% removal efficiency for COD. We were able to maintain better than 90% removal of COD by maintaining loading below 3 mg COD/L-d.

### Micronutrient and buffer addition study

#### Batch study

It has become apparent from recent advances in the application of anaerobic systems to industrial wastewater treatment that anaerobic organisms and in particular, methane forming bacteria have unique nutritional requirements. Since whey permeate is not a nutrient rich material, it was necessary to determine if it contains sufficient micro and macro nutrients to supply needs of the acid and methane forming bacteria in order to efficiently convert the organic matter in permeate to mostly methane, carbon dioxide and biological protein. Conclusions of this part of the study were as follows:

- 1) Addition of trace metals; Co, Ni, Mo, Fe resulted in inhibition of the methanogens
- 2) N & P in the permeate was not adequate and must be supplemented
- 3) pH control is the key to successful anaerobic digestion of whey permeate

### Quantity and quality of biological protein and methane produced

The amount of biogas produced depended on the loading rate as shown in Figure 1 and the amount of biological protein produced was most dependent on time since startup (Figure 2.). The quality of biogas was measured on the basis of percent methane, the higher the methane the better. The biological protein quality is based on protein content.

Figure 1 shows change in gas production with increased loading. The total gas produced increases at a predictable rate as indicated in Figure 1, however the methane produced per g COD destroyed did not change appreciably with loading rate. The methane produced per g COD destroyed was close to the theoretical maximum value of 0.35 L methane/g COD for the entire experiment. More total gas was produced at the higher loading rate, however the percent methane was reduced, making the gas less valuable for energy. At least 65% methane is recommended for use without pretreatment, in a boiler. At the higher loading rates the gas quality decreased to about 44% methane.

It is concluded that the UASB can produce about 15 g of solids/L-d. Unfortunately, most of these solids (69%) were not organic and therefore not useful for food or feed. The inorganic solids were probably due to precipitation of sodium bicarbonate in complex form. Large quantities (2.5 g/L permeate) of sodium bicarbonate had to be added to maintain a neutral pH in the digester.

Loading rate changed in a non linear fashion, but the rate of solids production was linear as shown in Figure 2. The rate of solids production did not respond to changes in loading rate. Solids production was dependent on time in a linear relationship. Apparently, the bacteria were reproducing at maximum possible rate at the lower (1 g COD/L-d) loading.

This project needs to be continued to learn how to control pH without adding expensive chemicals if this technology is to be economically viable. Our future work will investigate the addition of methanogens, loading rate, and increased sludge bed volume to control pH without chemicals.

### **Impact of project:**

Ultrafiltration of whey has potential of increasing demand and value of cheese whey. However, ultrafiltration of whey will continue to be suppressed until the disposal problem of whey permeate is solved.

A hypothetical but thorough economic analysis of processing whey by ultrafiltration to yield whey protein and then anaerobic digestion of permeate to produce methane, predicted a rate of return on the investment in processing equipment of 27.9% (Foth & Dyke Industrial, 1984).

### **Publications:**

\*Choudhury G.S., C.L.Hansen, Y. Bian, R. Dupont. 1989. Conditioning of Whey Permeate for Methane and Biomass Production Proceedings of the Fifth International Congress on Engineering and Food, May 28 June 3, 1989. Cologne, Germany.

\*Hansen, C.L., H.J. Xu. 1988. Metals Detoxification Using Whey Permeate. Proceedings of the 8th Biennial Cheese Industry Conference Aug 23-25, 1988. Logan, Ut. Invited.

\*Xu, H.J., C.L. Hansen, G.S. Choudhury. 1987. Using Dairy Byproduct for Mercury Waste Detoxification. Proceedings of the Food Processing Waste Conference Sept. 1-2, Atlanta, GA.

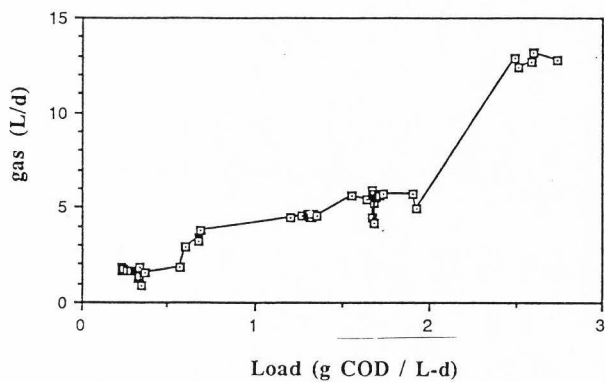


Figure 1. Gas production with increased organic loading

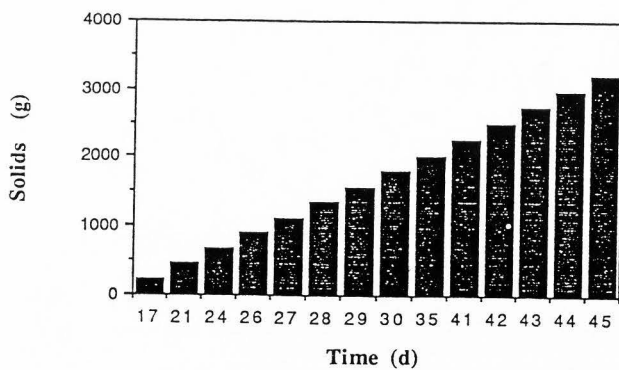


Figure 2. Solids production in the bioreactor

Project Outline

UTAH AGRICULTURAL EXPERIMENT STATION

Project No. \_\_\_\_\_

Fund: \_\_\_\_\_

Title: Continuous Production of Cottage Cheese from  
Ultrafiltrated Skim milk Retentate.

Personnel, L.V. Ogden, Brigham Young University and  
C.A. Ernststrom, Utah State University

Original Starting Date: September 1, 1987

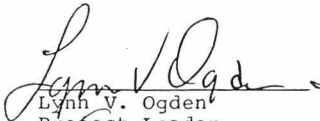
Duration of Current Project: Sept 1, 1987      August 31, 1988  
Starting Date      Estimated  
Completion Date

Institutional Units Involved: Brigham Young University,  
Food Science and Nutrition Dept.  
Utah State University  
Nutrition & Food Science Dept.

Authorized:

Brigham Young University

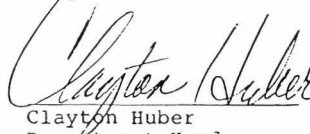
Utah State University

  
Lynn V. Ogden  
Project Leader

July 16 '87  
Date

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C.A. Ernststrom  
CoProject Leader

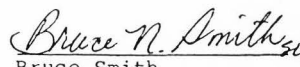
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Clayton Huber  
Department Head

July 16 '87  
Date

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Department Head

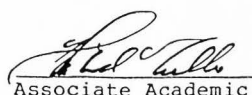
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Associate Academic  
Vice President  
BYU

7/17/87  
Date

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Director Ag Exp. Station Date

Continuous Production of Cottage Cheese  
from Ultrafiltered Skim Milk Retentate

L.V. Ogden  
Brigham Young University

and

C.A. Ernstrom  
Utah State University

Justification

Cottage cheese production in the United States is about one billion pounds annually (6) and is a very important dairy product in this country. Cottage cheese is produced by a traditional culturing method, (1) and also by direct acidification which was approved by FDA in 1969 (4). It is estimated that 25-30% of U.S. Cottage Cheese is produced by the direct acid method (6). Both methods as currently applied require the use of large vats.

Direct acid manufacture gives higher yields and avoids losses due to starter failure. A yield improvement of 11.1 and 8.7% over cottage cheese made with milk or whey based starter was reported by Geilman(5). Much of the economic effect of yield improvement is offset by high cost of acidulation. The direct acid method requires the use of expensive Glucono Delta Lactone (GDL) which stoutly converts to gluconic acid and coagulates the quiescent milk. Ingredient costs for directly acidified cottage cheese were 6.2% and 10.2% over costs of culturing with milk or whey based starters respectively.

The setting, cutting and cooking steps of cottage cheese manufacture by both methods have reached the limits of efficiency in conventional equipment. Vat sizes are as large as 40 to



50,000 pounds. Cross cutting of the curd is still a hand operation which limits the width and height of the vats. Large vats also result in long filling and draining times which add a non-productive hour or more to the production time. Larger vats would result in more non productive time.

Further efficiencies will require a continuous setting, cutting, and cooking process and could be further improved by pre concentrating by ultrafiltration.

#### Previous Work and Present Outlook

A continuous system for setting and cutting cottage cheese was developed by C.A. Ernstrom in 1965 (2). Cold 40°F milk was acidified to pH 4.5 - 4.7 with hydrochloric or phosphoric acid without coagulation occurring. Acidified milk was then warmed quiescently as it passed through 3/8 inch stainless steel tubes. The curd strand formed as the temperature rose. Whey expelled from the curd served as a lubricant between the curd strand and the tube wall which allowed the product to slip up the tube without agitation. When the curd strand emerged, it was cut to desired length by a rotating knife. The curd was then fed into a continuous cooker that cooked the curd in a relatively few minutes. Total process time from cold acidification to the end of washing was 20 to 25 minutes.

Dean Foods made cottage cheese by this process and sold it only in Illinois for about three years. Distribution restrictions forced Dean Foods to discontinue the process and remove the equipment. Inclusion of this process in the Federal Standard of Identity came soon after Dean Foods had removed their

equipment(3). The product made at Dean Foods was judged to be equal in consumer acceptability to cottage cheese made by the culturing process. We noticed, however, that the product was softer and absorbed cream more heavily than the traditional product.

Skim milk retentate has been successfully used to make cottage cheese by the direct acidification method using glucono-delta-lactone by Ocampo (8). He learned that 3x retentate was about the optimum concentration and that the firmness of the curd could be controlled by preheat treatment of the milk. Retentate that had received only a pasteurization treatment resulted in too firm a curd. Additional heat treatment was required to soften the curd to a desirable consistency.

The purpose of this study is to marry the technology developed for continuous production of cottage cheese with new technology for making direct acid curd from ultrafiltered skim milk retentate. Advantages of the proposed process would be (1) increased efficiency of acidification, curd formation and cooking (2) better sanitation in enclosed equipment, (3) reduced acid costs as compared to direct acidification in a vat and (4) the possibility of increased yield by elimination of washing.

### Procedures

#### Phase I

1. Skim milk ultrafiltrates 3x retentate of about 9.2% protein will be prepared.
  2. Retentate will be cold acidified with phosphoric acid.
- Preliminary trials by Ocampo have demonstrated that it can

be done without coagulation(9).

3. Curd will be formed in a tubular heat exchanger similar to the one described by Ernstrom in his development of the continuous process for skim milk(2).
4. After cutting, the curd will be cooked and washed in the traditional way in bulk.

#### Phase II

1. Study the effect of diafiltration as a means of removing lactose and eliminating the need for washing.
2. Study the effect of retentate heat treatments on yield and final curd texture.
3. Design and construct continuous cooker and determine temperature profile that will adequately cook the curd in minimum time.

#### Equipment Needs

The BYU Graduate Student working on this project will relocate to Logan and work intensively for approximately 6 months on this project. There he will have access to ultrafiltration equipment, a small plate heat exchanger for retentate heat treatment, and a continuous curd former.

#### References

1. Code of Federal Regulations, 1984, Title 21: 133.128
2. Ernstrom, C.A. 1965. Continuous process developed for making cottage cheese and without culture. Canadian Dairy and Ice Cream J. 44:(2)32.
3. Federal Register. 1970. 35:17110.
4. Federal Register. 1973. 38:6886.
5. Geilman, W.G. 1981. Comparison of skim milk starter, whey based starter and a direct set method on yield, quality and

- economics of cottage cheese production. M.S. Thesis. Utah State University, Logan.
6. Gemm, U. 1986 Carlin Foods, Inc. Personal communications.
  7. Milk Industry Foundation. 1985. Mil Facts p 28-9.
  8. Ocamps, J.R. 1987. Cottage cheese from ultrafiltered skim milk. J. Dairy Sci. 56 Supl p. 67 (abstract)
  9. Ocampo, J.R. 1987. Utah State University. Personal Communications.

### Financial Support

This project will be supported by Brigham Young University and the Western Dairy Foods Research Center. A project will be set up at BYU to pay the stipend and some supplies. The remainder of the supplies will be covered by a project set up at Utah State University.

### Budget

	<u>At BYU</u>	<u>At USU</u>
Research Assistant Stipend	8700.00	
Membranes		1000.00
Milk		2000.00
Supplies	<u>500.00</u>	<u>1500.00</u>
Total	9200.00	4500.00

### Source

BYU	5000.00	
Dairy Center Request	<u>4200.00</u>	<u>4500.00</u>
	9,200	4,500

**Project Title:** Continuous Production of Cottage Cheese from Ultrafiltered Skim Milk Retentate.

**Personnel:** L. V. Ogden, Associate Professor, Food Science and Nutrition, Brigham Young University

Rick Lord, Graduate Research Assistant, Food Science and Nutrition, Brigham Young University.

**Funding Sources:** Western Dairy Foods Research Center  
College of Biology and Agriculture  
Department of Food Science and Nutrition, Brigham Young University.

### Objectives:

Learn to make cottage cheese using a continuous process from ultrafiltered skim milk retentate while studying the effect of retentate concentration and several processing variables on properties of curd.

### Results:

The effect of processing variables on curd characteristics were investigated. Retentate concentration, retentate preheat treatment, pH of cold acidification prior ultrafiltration, pH of cold acidification prior to curd formation, acidification agents, cooking time and cooking temperature were investigated.

Curd formation experiments to date have been conducted quiescently in tubes. Attempts to form curd in tubes with the retentate flowing have not been successful. It appears equipment will have to be designed that will allow a brief quiet period as the curd forms.

Cottage cheese curd samples from each of the curd forming experiments along with commercial curd samples were evaluated by a panel of descriptive judges. Samples were rated as to their degree of firmness, mealiness, matting and off flavor. Data from the taste panels indicate that curd firmness increases with higher cooking temperatures. Maximum curd firmness at a given cook temperature is achieved by cooking for about 60 minutes. Firmness decreases slightly at shorter and longer cook times. Curd firmness is also affected by acidifying agent used. HCl or citric acid produces firmer curd than phosphoric or lactic acid.

Mealiness was a problem with this curd. It appears to be almost entirely dependent on cook temperature. As cook temperature is increased the degree of mealiness also increases. The amount of mealiness can be reduced somewhat by not acidifying the skim milk prior to ultrafiltration. Even then the resulting curd is significantly more mealy than commercial cottage cheese curd.

To date cottage cheese curd samples have been made that are similar to commercial cottage cheese in firmness, degree of matting and flavor, but are more mealy than commercial curd. Firmness and mealiness seem to come together. Soft curd can be made that lacks mealiness, but as firmness is increased mealiness also increases. More work must be done to reduce mealiness while retaining the desired degree of firmness.

**Impact of Research:**

Setting, cutting and cooking steps of cottage cheese manufacture by both the direct acid method and culturing methods have reached the limits of efficiency in conventional equipment. Vat sizes are as large as 40,000 to 50,000 pounds. Cross cutting of the curd is still a hand operation which limits the width and height of the vats. Large vats also result in long filling and draining times which add a non-productive hour or more to the production time. Larger vats would result in more non-productive time. Further efficiencies will require a continuous setting, cutting and cooking process and could be further improved by preconcentration by ultrafiltration. A totally enclosed continuous system would also protect the quality and shelf stability of the product.

**Project Title:** High Yield, low moisture cheese from homogenized UF milk.

**Personnel:** Donald J. McMahon, Assistant Professor, Department of Nutrition and Food Sciences, Utah State University

Brian J. Orme, Graduate Student, Department of Nutrition and Food Sciences, Utah State University

**Funding Sources:** Western Dairy Foods Research Center  
Utah State University Agricultural Experiment Station

### **Objectives:**

1. Determine effects of homogenization treatment on fat losses from UF retentate curd.
2. Design a cheese making process so as to obtain cheese in the range of pH 5.0 - 5.4 and moisture content < 40%.
3. Determine effects of milk heat treatment on moisture, texture and body of cheese made from UF retentate.
4. Provide a manufacturing procedure for making acceptable low moisture cheese from pre-fermented UF retentate that could be adopted for a continuous cheese making process.

### **Results:**

#### Objective 1:

Preliminary studies on the effects of homogenization on casein/fat complexing have been conducted. The laboratory homogenizer and heating systems used in these experiments, however, introduced too much variation into the results. This work is to be repeated using the newly installed Alfa Laval Sterilab UHT system to study the effect of temperature and homogenization pressure on the amount of casein/fat complexing that occurs during homogenization. The extent of this complexing will be determined by measuring the quantity of fat that sediments with the casein micelles during ultracentrifugation.

#### Objective 2:

Development of the correct curd structure is a vital part of cheese making. Cheese curd made from ultrafiltered milk retentate typically has poor textural properties. In order to duplicate the curd structure associated with cheese made from normal strength milk, we have conducted experiments to look at the relationships between milk concentration, pH, temperature, and enzyme concentration on the rate at which milk coagulates and forms a gel.

Enzymic coagulation time was found to be directly proportional to inverse of enzyme concentration at all retentate concentrations, pHs and temperatures studied but the proportionality constant varies widely with these parameters.

A series of charts included at the end of this report indicate that below pH 6.4, clotting time is not greatly affected by milk concentration, but at the natural pH of milk and concentrate (pH 6.7),

concentration has a greater effect. The graphs also show that increasing temperature produces an approximately second order decrease in coagulation time at all concentrations and pHs studied.

Rennet curds of ultrafiltration concentrated milk firm very fast and can become tough and uncuttable if not cut within a narrow window. Formagraph curves of 5X retentate show that retentates with higher pH (pH 6.7) and lower temperature firm more slowly and produce firming curves more like those of unconcentrated milk. Further study will determine if using lower setting temperatures with higher rennet levels will produce a curd in a reasonable time with better texture and syneresis.

### **Impact of Research:**

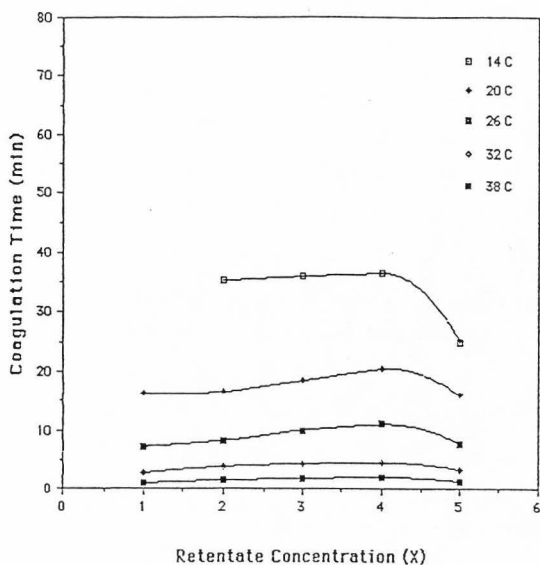
The technology of concentrating milk by ultrafiltration (UF) has progressed well over the past ten years. Much of this technology has been utilized in the development of new methodology for manufacture of cheese. The most successful applications have been in the production of high moisture cheeses by European based companies. Its has been of only limited use in the manufacture of low moisture cheeses because concentration of milk by ultrafiltration reaches a limit at approximately 40% total solids, i.e. a minimum of 60% moisture content .

In making a low moisture cheese from UF retentate texture and body is usually unsatisfactory for use as a value-added 'natural' cheese. It is our intention that through the development of a new cheese making procedure, as described in this research proposal, it will be possible to make low moisture cheeses that will retain the high yield advantages of ultrafiltration. Cheeses in the moisture range of 35-45% are the most widely consumed cheeses in the U.S.A. today. Of the total 1987 cheese production of 6.3 billion pounds, 42% was American cheese (2.7 billion pounds) and 30% was Italian cheese (1.8 billion pounds). From this it would appear that the introduction of new varieties of cheese would have the greatest success if they were in these categories.

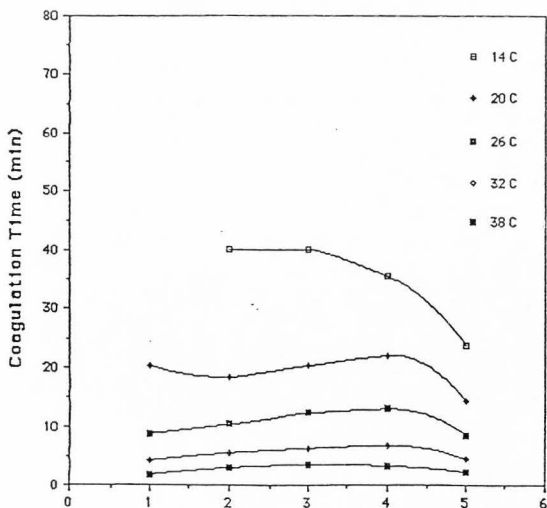
### **Publications:**

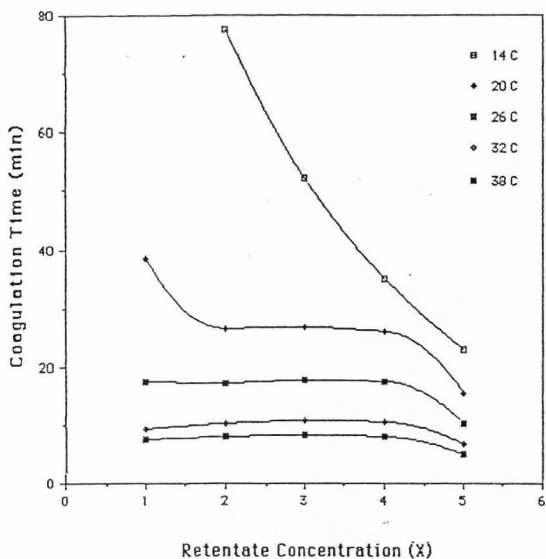
Orme, B. J. and D. J. McMahon. 1989. Effect of concentration on coagulation of ultrafiltered milk. J. Dairy Sci. 71 (Suppl. 1).



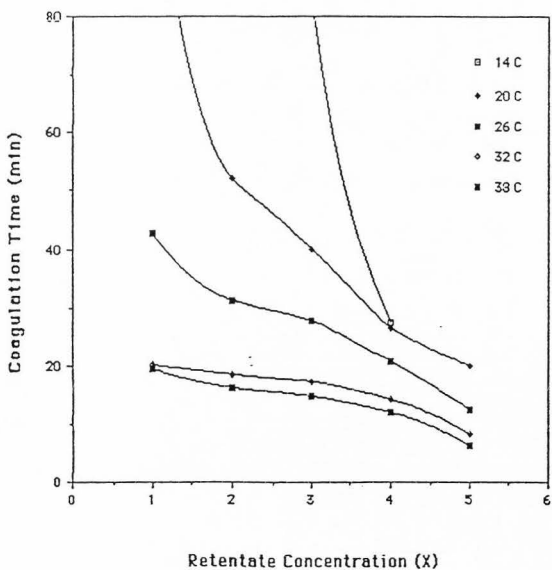


Coagulation Time vs. Milk Conc. @ pH 6.1





Coagulation Time vs. Milk Conc. @ pH 6.7



**Project Title:** High Yield, Low Moisture Cheese from Homogenized UF Milk

**Personnel:** Donald J. McMahon, Principal Investigator, Dept. of Nutrition and Food Sciences, Utah State University.

Brian Orme, Graduate Student, Dept. of NFS, USU.

**Funding Sources:** Western Dairy Foods Research Center  
Utah Agricultural Experiment Station

### Objectives:

Ultrafiltration (UF) of milk can be used for the manufacture of high moisture cheeses. Its economics lay mainly in increased retention of fat and protein. There are, however, some difficulties and complexities of making low moisture cheese using UF concentrated milk in that high fat losses occur and it is difficult to remove moisture. The specific objectives of this project are:

1. Determine effects of homogenization treatment on fat losses from UF retentate curd.
2. Design a cheese making process so as to obtain cheese in the range of pH 5.0—5.4 and moisture content < 40%.
3. Determine effects of milk heat treatment on moisture, texture and body of cheese made from UF retentate.
4. Provide a manufacturing procedure for making acceptable low moisture cheese from pre-fermented UF retentate that could be adopted for a continuous cheese making process.

### Results:

#### Objective 1: Homogenization and Fat Loss:

The high shear environment of UF, especially in pilot scale batch equipment, can damage natural milk fat globule membranes resulting in considerable whey fat when milk is coagulated and curds are cooked. Experiments have been conducted to determine effects of homogenizing whole milk on the extent of fat/casein complexing that occurs and the subsequent retention of fat in cheese curds made from 5X UF retentate.

In one experiment, pasteurized whole milk was divided into two lots and one lot homogenized at 3500 psi, while the other was not homogenized. Both lots were ultrafiltered without diafiltration to 38% solids. Cheeses were made simultaneously from 3.5 kilogram batches of retentate from each lot using standard cheddar make parameters. A one half kilogram, pH 5.0 water overlay, was used to float curds.

Over the course of agitating and cooking curds to 39°C, a 1 cm deep free-fat layer developed in whey from the unhomogenized sample, while whey from the

homogenized sample contained only a discontinuous film of free-fat. Final moisture levels were high, that is, in the range of 42—43% but homogenization did not significantly affect final moisture. On this visual basis, homogenization shows promise for increasing fat retention of UF retentates.

#### Objective 2: Cheese making Process

Our experiments have shown that less rennet is required to coagulate 5X retentates if pH is below 6.4. UF retentates are also much less viscous and more easily handled if they have been adjusted to pH 6.4—6.0. We have considered that if retentates were pre-fermented to pH 6.4 at approximately 30°C, temperature could then be reduced to 20°C to slow acid production and provide a larger make-window. If retentates were renneted and cut at 20°C, curds could be cooked over a 19C° range instead of a 9C° range to increase syneresis. Experiments to determine the effect of this procedure on cheese moisture are beginning.

#### Objective 3: Milk Heat Treatment

Work on this objective will not be undertaken until objectives 2 and 3 are completed.

#### Objective 4: Cheese Quality

Textures and microstructures of cheese made from UF retentate differ from those of traditionally-made cheese. Deviations in curd microstructure are believed to result in part from effects of concentration on the coagulation process. Coagulation of milk is a two phase process. Enzymic hydrolysis of k-casein is followed by aggregation of casein particles. However, these two phases overlap. Studies made in our laboratory have shown that coagulation of milk normally occurs when hydrolysis of k-casein is 80—90% complete. However, when milk is concentrated to 5X, coagulation occurs when only 50—60% of k-casein is cleaved. This compression of the enzymic and aggregation phases of coagulation may in part be responsible for different curd structure and final properties of UF cheese.

By lowering coagulation temperature, coagulation is slowed far more than can be accounted for simply by decreased enzyme activity. Rather there is a marked slowing of aggregation rate caused by lowering temperature. This slowing of aggregation rate relative to proteolysis should provide a more natural curd texture in the final cheese.

#### **Impact of Research:**

The technology of concentrating milk by ultrafiltration (UF) has progressed over the past ten years. Much of this technology has been utilized in the development of new methodology for manufacture of cheese. The most successful applications have been in the production of high moisture cheeses by European companies. However, UF has been of only limited use in the manufacture of low moisture cheeses because concentration of milk in most UF systems reaches a limit at approximately 40% total solids, or in other words it reaches a minimum of 60% moisture content. The UF retentate thus requires further processing in order to reduce its moisture content to an acceptable

range for many of the more popular cheese types in the U.S.A.

In making a low moisture cheese from UF retentate there have been two methods used to extract moisture from the cheese curd. Vacuum evaporation has been used for the production of a cheese base for use in process cheese. It, however, produces a product with texture and body unsatisfactory for a value-added 'natural' cheese.

A method has been developed for using UF retentate to make Cheddar cheese by passing UF curd through a mechanical syneresis system followed by a mechanical cheddaring system. This is successful in lowering cheese moisture but suffers from fat losses and thus loses much of the yield advantage of using UF.

It is our intention that through the development of a new cheesemaking procedure, as described in this research project, it will be possible to make low moisture cheeses that will retain the high yield advantages of UF. Cheeses in the moisture range of 35-45% are the most widely consumed cheeses in the U.S.A. The introduction of new varieties of cheese would have the greatest opportunity for success if they were in this category.

Cheesemaking technology to be developed in this project has the potential to provide opportunities to produce new low moisture cheese products on a cost effective basis.

#### **Publications and Abstracts:**

B.J. Orme and D.J. McMahon. 1989. Effect of concentration on coagulation of ultrafiltered milk. 84th American Dairy Science Association Meeting, J. Dairy Sci:Supp. 1, 184.

## High Yield, Low Moisture Cheese from Homogenized UF Milk

**Annual Report Date:** 30 June 91    **Project Term:** 1 Sep 88 — 31 Aug 91

### Personnel:

Principal Investigator: Dr. D.J. McMahon  
Graduate Student: Mr. Brian Orme

**Funding Sources:** Western Dairy Foods Research Center  
Utah Agricultural Experiment Station

### Objectives:

Ultrafiltration (UF) of milk can be used for the manufacture of high moisture cheeses. Its economics lay mainly in increased retention of fat and protein. There are, however, some difficulties and complexities of making low moisture cheese using UF concentrated milk in that high fat losses occur and it is difficult to remove moisture. The specific objectives of this project are:

1. Determine effects of homogenization treatment on fat losses from UF retentate curd.
2. Design a cheese making process so as to obtain cheese in the range of pH 5.0—5.4 and moisture content < 40%.
3. Determine effects of milk heat treatment on moisture, texture and body of cheese made from UF retentate.
4. Provide a manufacturing procedure for making acceptable low moisture cheese from pre-fermented UF retentate that could be adopted for a continuous cheese making process.

### Results:

#### Objective 1: Homogenization and Fat Loss.

Experiments have been conducted to determine effects of homogenizing whole milk on the extent of fat/casein complexing that occurs and the subsequent retention of fat in cheese curds made from 5X UF retentate.

In one experiment, pasteurized whole milk was divided into two lots and one lot homogenized at 3500 psi, while the other was not homogenized. Both lots were ultrafiltered without diafiltration to 38% solids. Cheeses were made simultaneously from 3.5 kilogram batches of retentate from each lot using standard cheddar make parameters. A one half kilogram, pH 5.0 water overlay, was used to float curds.

Over the course of agitating and cooking curds to 39°C, a 1 cm deep free-fat layer developed in whey from the unhomogenized sample, while whey from the homogenized sample contained only a discontinuous film of free-fat. The extent of fat/protein complexing has been determined over a range of homogenization temperatures and pressures.

#### Objective 2: Cheese making Process

5X UF Retentate. Our experiments have shown that less rennet is required to coagulate 5X retentates if pH is below 6.4. UF retentates are also much less viscous and more easily handled if they have been adjusted to pH 6.4—6.0.

We have considered that if retentates were pre-fermented to pH 6.4 at approximately 30°C, temperature could then be reduced to 20°C to slow acid production and provide a larger make-window. If retentates were renneted and cut at 20°C, curds could be cooked over a 19°C range instead of a 9°C range to increase syneresis.

4X UF Retentate. To overcome the processing difficulties in using 5X retentate we have looked at the use of 4X UF retentate as the starting material for cheesemaking. Diafiltration levels required to reduce lactose to a level so that fermentation stops at pH 5.1 during cheesemaking have been determined.

#### Objective 3: Milk Heat Treatment

Work on this objective has been delayed until an appropriate cheesemaking procedure has been determined.

#### Objective 4: Cheese Quality

Cheese quality is affected by the microorganisms present in the cheese curd. Unpredictable fermentation rates have been observed when UF retentate is used to make cheese. We studied the functioning of a variety of strains of *Lactococcus lactis* ssp *lactis* and ssp *cremoris* in 4X retentate and found that there are significant strain differences in how they perform. The growth of some strains is inhibited in UF retentate while the generation time of other strains was not affected.

#### **Impact of Research:**

The technology of concentrating milk by ultrafiltration (UF) has progressed over the past ten years. Much of this technology has been utilized in the development of new methodology for manufacture of cheese. The most successful applications have been in the production of high moisture cheeses by European companies. However, UF has been of only limited use in the manufacture of low moisture cheeses because concentration of milk in most UF systems reaches a limit at approximately 40% total solids, or in other words it reaches a minimum of 60% moisture content.

It is our intention that through the development of a new cheesemaking procedure, as described in this research project, it will be possible to make low moisture cheeses that will retain the high yield advantages of UF. Cheeses in the moisture range of 35-45% are the most widely consumed cheeses in the U.S.A. The introduction of new varieties of cheese would have the greatest opportunity for success if they were in this category.

Cheesemaking technology to be developed in this project has the potential to provide opportunities to produce new low moisture cheese products on a cost effective basis.

#### **Publications and Abstracts:**

McMahon, D.J. and R.J. Brown. 1990. Development of surface functionality of casein particles as the controlling parameter of enzymic milk coagulation. *Colloids and Surfaces*. 44:263-279.

## PROPERTIES OF LOW-FAT YOGURT MANUFACTURED FROM ULTRAFILTERED AND ULTRA-HIGH TEMPERATURE TREATED MILK

Personnel: Paul A. Savello, Assistant Professor, Utah State University  
Richard Dargan, Graduate Assistant, Utah State University

### JUSTIFICATION:

Yogurt production using ultrafiltration (UF) technology has only been researched and reported. Chapman et al. (2) produced yogurt of 21% total solids after UF. The product was comparable in flavor, body and texture to yogurt produced with milk fortified with nonfat dry milk solids. Abrahamsen and Holmen (1) ultrafiltered milk to produce yogurt that had a firmer coagulum and higher viscosity than a non-UF control. The acidity of the UF sample was more distinct.

Yogurt production using ultra-high temperature (UHT) treatments of the milk has also been reported. Schmidt et al. (8) and Labropoulos (5) reported that UHT treatment of yogurt milk produced a final product with lower firmness, lower viscosity and less syneresis. Labropoulos also reported that maximum whey protein denaturation (88%) followed a UHT treatment of 149° C for 3.3 sec compared to the same maximum whey protein denaturation with a vat treatment of 82° C for 5 min.

As UF concentration of yogurt milk gives a final product with firmer body and higher viscosity while UHT treatment of yogurt milk yields a product of lower firmness and reduced viscosity, the two technologies can prove beneficial to the production of yogurt with improved qualities. As UHT treatment of yogurt milk provides reduced syneresis in the final product, the combination of UF and UHT treatments can also yield a more consistent product with wider consumer appeal.

The advantages (including economic) of UF concentration and UHT treatment of lowfat milk for yogurt manufacture include:

- a. increase of total milk solids without addition of dried non-fat milk solids;
- b. control of yogurt milk quality by UF concentration prior to culture inoculation due to fewer dry ingredients being added to the yogurt milk;
- c. improved product quality with reduced fat levels;
- d. reduced heat input by UHT treatment compared to longer vat holding times to denature whey proteins;
- e. reduced inventory of added ingredients, including non-fat milk powder and stabilizers.

These advantages can prove beneficial if the yogurt produced is of acceptable quality. The product quality can be measured by flavor/texture, acidification time to gelation, and minimum defects as syneresis, graininess, and flocculation.

UF technology can provide a means to control desired final product quality. UF concentration to different total milk solids levels together with different heat treatments prior to inoculation can indicate the relationships between milk solids/heat treatment to give different (yet acceptable) yogurt products.



## PREVIOUS WORK/PRESENT OUTLOOK:

Chapman et al. (2) produced an acceptable yogurt product by concentrating milk to 18-20% total solids. The product was judged as acceptable with good flavor and texture. No objective measurements of viscosity, rheologic properties or gel strength were indicated in the report.

Yogurt milk that has been heat treated by vat, HTST, and UHT procedures with subsequent impacts on flavor and texture has been reported (4-8). Flavor profiles of the differently heated yogurt milks were not significantly different (4) whereas the textures of finished yogurts were different (6,8). The heat treatment of yogurt milk, therefore, plays a major role in the final body/texture of the product.

Combining UF concentration and different heat treatments to the yogurt milk can provide a profile to understand the effect(s) of each process as it relates to yogurt quality. By concentrating to the desired total milk solids level by UF without addition of external dried milk solids, more carefully controlled objective measurements can be made of body/texture.

## OBJECTIVES:

The objectives of this research proposal are:

1. To measure the effects on the yogurt properties of viscosity, gel strength, syneresis, and water holding capacity by ultrafiltering yogurt milk to different total milk solids levels and applying different heat treatments;
2. To observe structural differences by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of the differently treated yogurt milks;
3. To measure the acceptability of yogurt flavor and body/texture by appropriate taste panel procedures;
4. To measure the effect on acidification time to desired gelation level by different heat treatments;
5. To measure the whey protein denaturation in the yogurt milk as a result of the different heat treatments.

## PROCEDURES:

1. UF of yogurt milk. Milk will be ultrafiltered (batch-wise) to total milk solids of 14, 16 and 18%. The final yogurt milk will contain 0.5 to 2% milkfat.
2. Heat treatments of UF yogurt milk. The yogurt milk will be heat treated as follows:
  - a. Vat: 82 C for 20 min.
  - b. HTST: 95 C for 1.5 min.
  - c. UHT: 140 C for 6, 10, 20 sec.
3. Physical properties measurements.
  - a. Viscosity. Brookfield viscosity measurements will be conducted of the finished yogurt products;
  - b. Gel strength. Penetrometer depth measurements of appropriate time duration will indicate yogurt gel strength.
  - c. Syneresis. Syneresis measurements will be conducted according to Schmidt et al. (8). Surface whey is removed by aspiration after inclining yogurt samples 90 degrees.
  - d. Water holding capacity. Water holding capacity (WHC) of yogurt is determined according to Kalab et al. (3). The procedure involves centrifugation of the yogurt sample with subsequent weighing of the pellet. The WHC can be expressed as percent pellet weight relative to the yogurt sample weight.
  - e. Acidification time of the yogurt samples will be determined by monitoring acid development (i.e. pH) over the incubation time required to reach pH 4.9-5.0.
  - f. Flavor/Texture/Body. These properties of the finished yogurt samples will be evaluated by selected faculty members and graduate students. Proper descriptions of flavor qualities, texture and body qualities and defects will be prepared and given to panelists prior to taste panels.
  - g. Whey protein denaturation. Denatured whey protein will be determined by precipitation of casein at pH 4.6 followed by denatured whey protein determination by saturated salt precipitation.

**Project Title:** Properties of Low-Fat Yogurt Manufactured From Ultrafiltered and Ultra-High Temperature Treated Milk

**Personnel:** Paul A. Savello, Assistant Professor, Nutrition and Food Sciences, Utah State University

Richard Dargan, Graduate Assistant, Nutrition and Food Sciences, Utah State University

**Funding Sources:** Western Dairy Foods Research Center  
Utah Agricultural Experiment Station

### Objectives:

The objectives of this research proposal are:

1. To measure the effects on the yogurt properties of viscosity, gel strength, syneresis, and water holding capacity by ultrafiltering yogurt milk to different total milk solids levels and applying different heat treatments;
2. To observe structural differences by scanning electron microscopy (SEM) and transmission electron microscopy (IEM) of the differently treated yogurt milks;
3. To measure the acceptability of yogurt flavor and body/texture by appropriate taste panel procedures;
4. To measure the effect on acidification time to desired gelation level by different heat treatments;
5. To measure the whey protein denaturation in the yogurt milk as a result of the different heat treatments.

### Results:

New project. UHT equipment has just become operational. Results will be presented in next years project report.

### Impact of Research:

Yogurt production using ultrafiltration (UF) technology has only been researched and reported. Chapman et al. (2) produced yogurt of 21% total solids after UF. The product was comparable in flavor, body and texture to yogurt produced with milk fortified with nonfat dry milk solids. Abrahamsen and Holmen (1) ultrafiltered milk to produce yogurt that had a firmer coagulum and higher viscosity than a non-UF control. The acidity of the UF sample was more distinct.

Yogurt production using ultra-high temperature (UHT) treatments of the milk has also been reported. Schmidt et al. (8) and Labropoulos (5) reported that UHT treatment of yogurt milk produced a final product with lower firmness, lower viscosity and less syneresis. Labropoulos also reported that maximum whey protein denaturation (88%) followed a UHT treatment of 149°

C for 3.3 sec compared to the same maximum whey protein denaturation with a vat treatment of 82° C for 5 min.

As UF concentration of yogurt milk gives a final product with firmer body and higher viscosity while UHT treatment of yogurt milk yields a product of lower firmness and reduced viscosity, the two technologies can prove beneficial to the production of yogurt with improved qualities. As UHT treatment of yogurt milk provides reduced syneresis in the final product, the combination of UF and UHT treatments can also yield a more consistent product with wider consumer appeal.

The advantages (including economic) of UF concentration and UHT treatment of lowfat milk for yogurt manufacture include:

- a. increase of total milk solids without addition of dried non-fat milk solids;
- b. control of yogurt milk quality by UF concentration prior to culture inoculation due to fewer dry ingredients being added to the yogurt milk;
- c. improved product quality with reduced fat levels;
- d. reduced heat input by UHT treatment compared to longer vat holding times to denature whey proteins;
- e. reduced inventory of added ingredients, including non-fat milk powder and stabilizers.

These advantages can prove beneficial if the yogurt produced is of acceptable quality. The product quality can be measured by flavor/texture, acidification time to gelation, and minimum defects as syneresis, graininess, and flocculation.

UF technology can provide a means to control desired final product quality. UF concentration to different total milk solids levels together with different heat treatments prior to inoculation can indicate the relationships between milk solids/heat treatment to give different (yet acceptable) yogurt products.

**Project Title:** Properties of Low-Fat Yogurt Manufactured From Ultrafiltered and Ultra-High Temperature Treated Milk

**Personnel:** Paul A. Savello, Principal Investigator, Assistant Professor, Dept. of Nutrition and Food Sciences, Utah State University

Richard Dargan, Graduate Student, Dept. of Nutrition and Food Sciences, Utah State University.

**Funding Source:** Western Dairy Foods Research Center  
Utah State University

## **Objectives and Results:**

**Objective 1:** To measure the effects on the yogurt properties of viscosity, gel strength, syneresis, and water holding capacity (WHC) by ultrafiltering to different total milk solids levels and applying different heat treatments.

**Materials and Methods:** Skim milk, 1% fat milk, skim milk ultrafiltered to 12.5% solids, and skim milk ultrafiltered to 15% solids were ultra-high temperature (UHT) treated by means of an Alfa-Laval Sterilab to 220, 240, 260, or 280°F for 4 sec. All milks except the 1% fat milk were also UHT treated for 8, 12, or 16 sec at each of the above temperatures. Control yogurts were prepared by vat pasteurization of aliquots of the same milks. Additional controls were prepared by vat pasteurization of skim milk with 2, 3, 4, or 5% added nonfat dry milk (NFDM) as a "traditional process" comparison. Vat heat treatment was 182°F for 20 minutes. Forty minute vat heated samples were also prepared for 2, 3, 4, or 5% added NFDM and 12.5% UF solids skim milks.

Measurements of viscosity, gel strength, syneresis, and water holding capacity were made on samples from all treatments at 7, 14, and 21 days.

**Viscosity.** Stirred viscosity measurements were made by Brookfield viscometer at 3 rpm with T-bar spindles. Following 30 seconds stirring, readings were taken every 30 seconds. An average of 4 readings was calculated for each sample. Four samples were tested per treatment.

**Gel strength.** A device was manufactured to give a relative measure of gel strength. A 5/8 inch shaft was lowered at a fixed speed into 100 ml samples on top of an analytical balance. Breaking force (gel strength) was taken to be the first point at which the balance reading stops increasing and decreases sharply. Four samples were determined per treatment.

**Syneresis.** Syneresis was measured by aspirating the whey off of the samples when inclined at a 90° angle. Four samples were measured for each treatment.

**Water holding capacity.** Water holding capacity (WHC) was measured by centrifuging approximately 40 grams of sample at 10,500 rpm for 30 minutes at 10°C. The supernatant was removed by draining the tube in an inverted position for 10 min. WHC was expressed as percent pellet weight relative to sample weight. Two samples were measured for each treatment.

**Results:** Viscosity of yogurt increased with increased UF solids (Figure 1). Viscosity of UHT yogurt was greater than the viscosity of yogurt from vat heated skim milk yogurt with comparable total solids from added NFDM. Viscosity did not increase directly with increased UHT temperature. Rather, viscosity was greatest from lower (220 and 240°F) temperature treatments. At 280°F, viscosity was reduced compared to other UHT temperatures. Viscosity tended to decrease with increasing UHT temperature at higher solids levels (12.5 and 15% UF solids). Increased holding time at 280°F increased viscosity.

Gel strength increased with increasing level of skim milk solids as a result of ultrafiltration. Gel strength was higher in yogurt made from UF/UHT milks than in vat heated yogurt made with added NFDM to even higher levels of solids (Figure 2). UHT yogurt from skim milk with 1% added milkfat had lower gel strength than yogurt from skim milk alone. Gel strength did not seem to improve directly with increased UHT temperature nor did holding time dramatically increase gel strength. Temperature, however, had more impact on gel strength than did holding time. 280°F had an unfavorable impact on gel strength at all solids levels. In most cases the lower temperatures (220 and 240°F) showed greatest gel strength. Twenty minute vat heat treatment of UF milks resulted in higher gel strength than any UHT treatment.

Higher UF solids decreased syneresis by more than would be expected by increased solids alone based on comparison to vat-heated yogurt milks with added NFDM (Figure 3). Addition of 1% milkfat also reduced syneresis. Increased UHT temperature increased syneresis in skim milk yogurt but the effect seemed to be overcome by the increased solids in the UF samples. Increased holding time had a pronounced effect on reducing syneresis of skim milk yogurt from the 280°F treatment, but had no effect on the already low syneresis in the higher solids yogurts (Figure 4). Syneresis was higher on vat treated yogurt than UHT yogurt.

Water holding capacity increased with increased level of skim milk solids as a result of ultrafiltration. The added solids from UF had a greater impact on increasing WHC than did comparable solids levels from addition of NFDM in a traditional (vat) heating process (see Figure 5). Yogurt made from 1% fat milk also had greater WHC than did skim milk yogurt. WHC did not increase with increasing UHT temperature. Rather, lower (220 and 240°F) temperatures seemed to provide the greatest WHC. Increased holding time did not improve WHC and, in some cases, reduced it.

**Conclusions:** Using ultrafiltration to increase solids affords greater viscosity, gel strength, WHC, and lower syneresis in UHT yogurt as compared to vat heated yogurt with added NFDM to comparable solids levels. Increasing UHT temperature (especially 280°F) does not improve gel strength, viscosity, WHC, and syneresis. Rather, lower temperatures (220 and 240°F) provide the greatest viscosity, gel strength, WHC, and least syneresis (especially when the solids are increased by UF). Intermediate UHT treatment may provide comparable or better WHC, and improved syneresis as compared to vatheated yogurt, but this effect becomes less distinguishable as solids are increased by UF (both heating methods are good). However, UHT treatment does not appear to be able to match the same levels of gel strength and viscosity that can be seen with vat heat treatment.

**Objective 2:** To observe structural differences by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of the differently treated yogurt milks.

- Incomplete to-date -

**Objective 3:** To measure acceptability of yogurt flavor and texture by appropriate taste panel procedures.

- Incomplete to-date -

**Objective 4:** To measure the effect on acidification time to desired gelation level by different heat treatments.

- Incomplete to-date -

**Objective 5:** To measure whey protein denaturation in the yogurt milk as a result of the different heat treatments.

**Materials and Methods:** Yogurt milks were prepared as in Objective 1. Unfermented samples of all heat treatments were tested for whey protein denaturation (WPD). Milk samples were adjusted to pH 4.6 by addition of 0.1N HCl. The precipitated casein was filtered out and the whey-protein-containing filtrate was collected. Kjeldahl nitrogen analysis was performed on the filtrate samples. The whey protein level found in the heat treated samples was compared to the whey protein level found in the raw milk of the same concentration. Whey protein denaturation was calculated as the disappearance of the whey protein in heat treated samples as compared to unheated controls as a result of whey protein-casein complexation.

**Results:** Yogurt milks with higher solids from UF had higher levels of WPD at each heat treatment (Figure 6). WPD increased with increasing UHT temperature and holding time. The effect of temperature was more dramatic at lower holding time (Figure 7). Temperature had a more dramatic effect on WPD than did holding time (except for holding time at 220°F). Vat heat treatment

resulted in WPD that was at or near the same level achieved by the most severe UHT treatments. It is interesting to note, however, that the highest WPD in UHT treatments did not correlate with the best yogurt properties (see Objective 1 Results). In fact, inferior viscosity, gel strength, WHC, and syneresis resulted at 280°F.

**Conclusions:** Whey protein denaturation may not be a good indicator of UHT yogurt properties. Although UHT treatments involve high temperatures, the resulting WPD is not as great as seen with lower temperature, longer time vat heating methods.

**Impact of Research:** This research indicates combining the technologies of ultrafiltration and ultra-high temperature treatment of yogurt milk can produce comparable or superior yogurt than traditional (vat heat treatment) yogurt of comparable total solids levels. The research has thus far indicated that the highest UHT temperatures of yogurt milk do not yield improved product characteristics over "lower" UHT temperatures.

Increasing the total milk solids by ultrafiltration appears to be a good means for improved yogurt physical characteristics. Measurements of these yogurt flavor and texture attributes must still be studied.

#### **Abstracts:**

Dargan, R.A., and P.A. Savello. 1990. Ultra-high temperature treatment of skim milk and low fat milk for yogurt. Poster Session. ADSA Annual Meeting, NC State.

Dargan, R.A., and P.A. Savello. 1990. Yogurt with improved physical properties from ultrafiltered and ultra-high temperature treated skim milk. Oral presentation. ADSA Annual Meeting, NC State.

#### **Patents:**

None to date.



Figure 1. Viscosity vs Heat Treatment

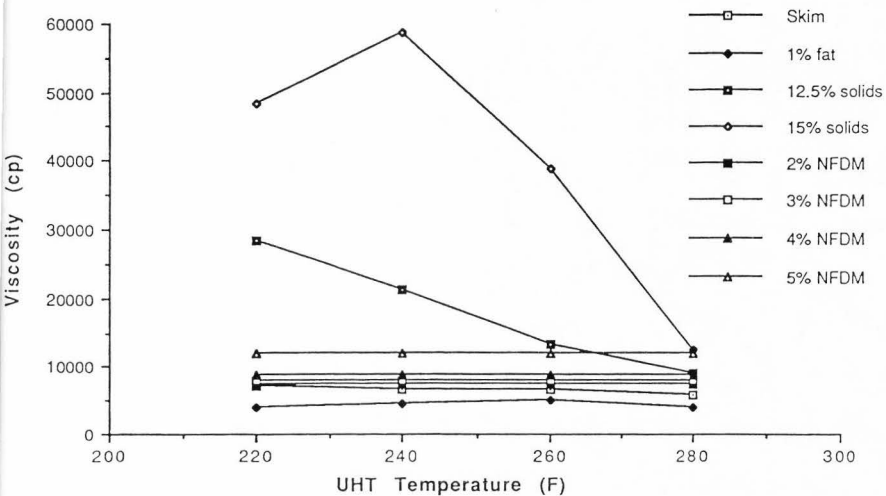


Figure 3. Syneresis vs Heat Treatment

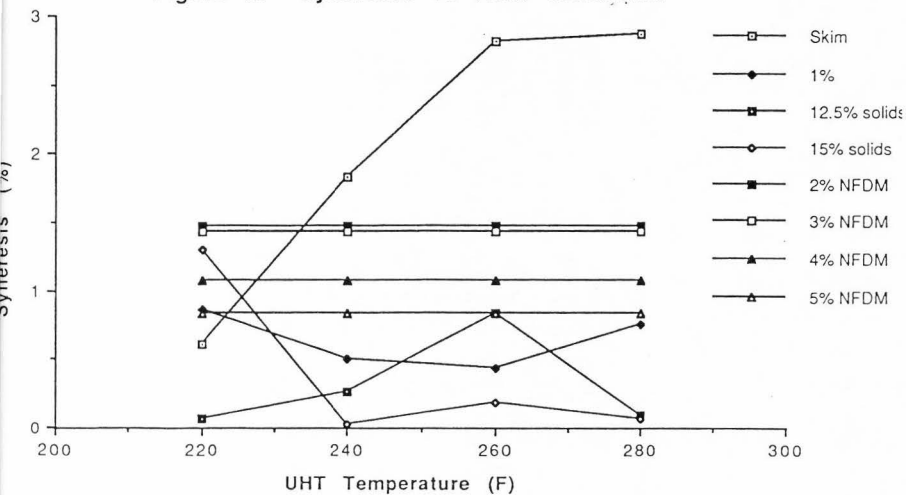


Figure 4. Syneresis vs Holding Time @ 280 F

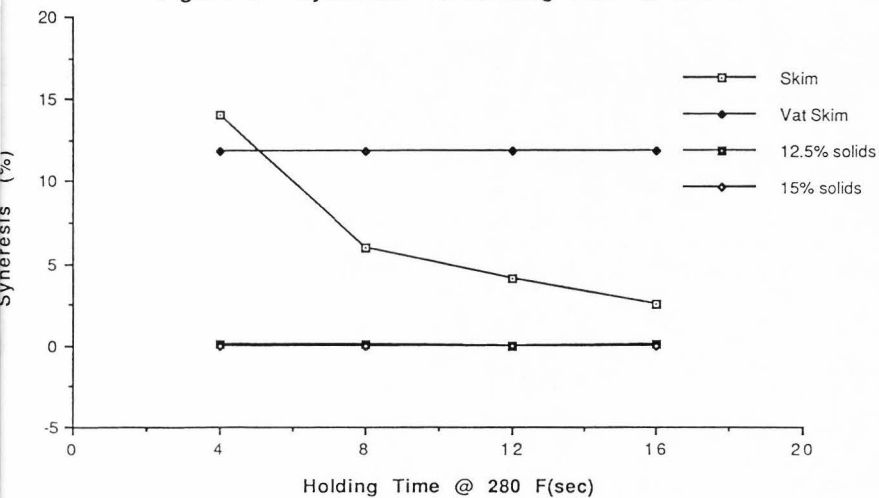


Figure 5. Water Holding Capacity vs UHT Treatment

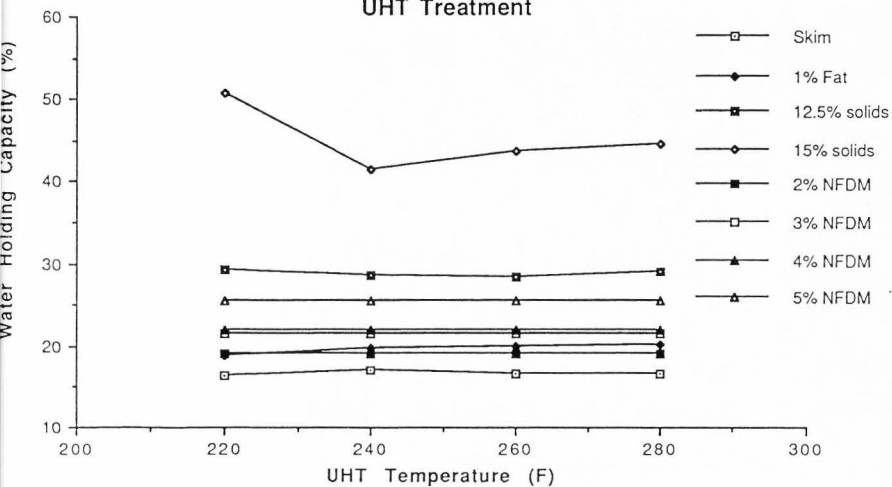


Figure 6. Whey Protein Denaturation vs Solids

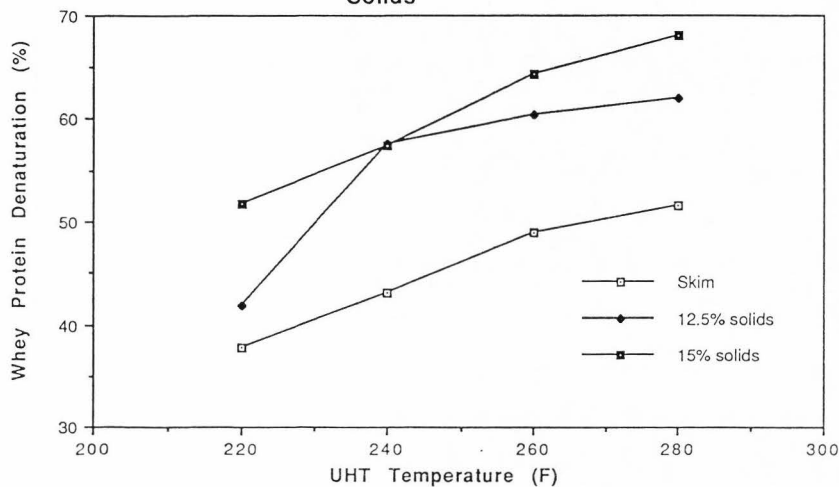
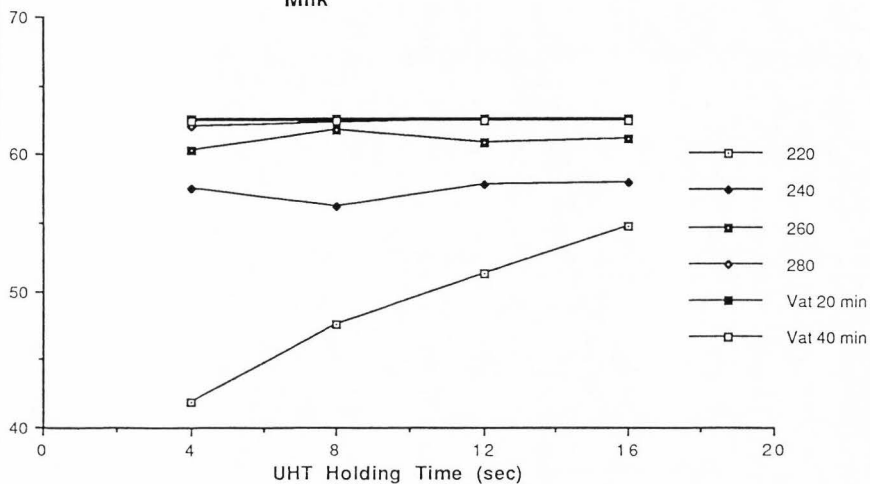


Figure 7. WPD of 12.5% UF Solids Yogurt Milk



**Project Title:** Properties of Low-Fat Yogurt Manufactured from Ultrafiltered and Ultra-High Temperature Treated Milk

**Personnel:** Paul A. Savello, Dept. of Nutrition and Food Sciences, Utah State University.

**Funding:** Western Dairy Foods Research Center

**Objectives:**

1. To measure the effects on the yogurt properties of viscosity, gel strength, syneresis, and water holding capacity by ultrafiltering yogurt milk to different total milk solids or standardized 5% protein level and applying different heat treatments.
2. To measure the whey protein denaturation in the yogurt milk as a result of different heat treatments.

**Results:**

Intermediate-(IHT) to ultra-high temperature (UHT) heat treatments were compared to vat heat treatment of skim milk, ultrafiltered (UF) skim milk, and skim milk with added nonfat dry milk (NDM) for yogurt. Yogurt made from skim milk ultrafiltered to 5% protein had greater gel strength and viscosity than yogurts enriched to 5% protein by NDM addition. This effect was observed for all heat treatments, including indirect plate exchange heating at 100, 110, 120, 130, or 140°C for 4 or 16 seconds, or vat heating at 82°C for 20 minutes. Vat heated UF yogurts had lower syneresis than vat heated NDM yogurts. IHT treatment (100°C for 16 sec, 110°C for 4 or 16 sec, 120°C for 4 sec) showed highest gel strength, viscosity, WHC, and lowest syneresis compared to high-end temperatures in all yogurts. UHT treatment (140°C for 4 or 16 sec) was detrimental to textural properties of skim milk, NDM, and UF yogurts.

In trials to produce an aseptic yogurt using UHT technology, pectin addition from 0.2 to 1.5% and temperatures as low as 100°C did not successfully stabilize the low pH (4.2-4.6) yogurts. Most promising results were received from yogurt milks that were H<sub>2</sub>O<sub>2</sub>-treated rather than prior pasteurization. As aseptic yogurt could provide new non-refrigerated sales opportunities for dairy products.

**Impact of Research:**

Previous reports on this project indicated that yogurt made from skim milk ultrafiltered (UF) to increase total solids resulted in greater viscosity, gel strength, water holding capacity (WHC), and lower syneresis in IHT yogurts (104, 116, 127°C for 4 sec)

compared to vat heated yogurts with added NFDM to comparable solids levels. Results presented here suggest that ultrafiltration also provides enhanced yogurt physical properties when used to enrich yogurt milks to a standardized 5% protein level as compared to traditional enrichment to 5% protein by addition of NDM. The use of UF to fortify yogurt may offer improved textural properties at lower total solids levels than traditional fortification, and potentially lower cost.

Previously reported data on skim milk, skim milk adjusted to 1% milkfat, and 12.5 and 15% solids UF skim milk indicated that IHT treatment of milk for yogurt optimizes the yogurt physical properties that can be achieved from indirect heat treatments between 104 to 138°C. If indirect plate heat exchange is to be used to process yogurt milks, results from 5% protein level data suggest IHT temperatures (100°C for 16 sec, 110°C for 4 or 16 sec, or 120°C for 4 sec) provide comparable or better yogurt properties in this study were achieved without the use of stabilizers. The use of UF and IHT technology both represent non-additive approaches to enhance yogurt physical properties.

#### **Publications/Abstracts:**

Dargan, R.A., and P.A. Savello. 1991. Protein Enrichment via Ultrafiltration Improves Yogurt Physical Properties. J. Dairy Sci. 74 (suppl. 1) (To be presented ADSA 1991).

Dargan, R.A., and P.A. Savello. 1990. Yogurt with improved physical properties from ultrafiltered and ultra-high temperature treated skim milk. J. Dairy Sci. 73:94 (suppl. 1).

Dargan, R.A., and P.A. Savello. 1990. Ultra-high temperature treatment of skim milk and low fat milk for yogurt. J. Dairy Sci. 73:111 (suppl. 1).



**Project Title:** Development of a process for production of UF milk retentate

**Personnel:** Conly L. Hansen, Dept. of Nutrition & Food Sciences, Utah State University

Donald J. McMahon, Dept. of Nutrition & Food Sciences, Utah State University

Yehia A. El-Samragy, Dept. of Nutrition & Food Sciences, Utah State University

**Funding:** Western Dairy Foods Research Center

### **Background:**

Several methods are available for processing surplus milk to extend its shelf life. Skim milk powder has been the standard means for storing surplus milk solids. Recently a frozen concentrate has also been developed (U.S. Patent 4,844,923). The major problem associated with frozen milk concentrate is the gradual destabilization of casein micelles that occurs during storage. Successful ultra-high temperature (UHT) concentrated milk has not yet been achieved because of the gelation that occurs in such concentrates upon storage of room temperature.

Production of milk powder by evaporative concentration and drying is an established segment of the dairy industry. However, product quality considerations favor the use of membrane separation to concentrate milk rather than thermal processes. Extensive heating during evaporative concentration often causes product degradation, primarily through change of color and flavors, and high denaturation of protein.

Milk powder with better functional and nutritional quality can be produced employing membrane separation such as ultrafiltration for concentrating milk.

### **Objectives:**

The overall objective of this project is to develop a process for production of high protein UF milk retentate powder.

### **Specific Objectives:**

1. Determining effects of heat treatment, pH and limited enzyme treatment of UF retentate on the functional properties of its resultant spray dried, high protein, milk powder.

2. Determining effects of drying parameters, such as particle size temperature, solid concentration and foam spray, on properties of the retentate powder.
3. Evaluating product applications of the high protein milk powder.

#### GANTT CHART

Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																						
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#### **Effect of different pH and heat treatments on the properties of UF milk retentate powder:**

Fresh raw skim milk was ultrafiltrated to 20% total solids. The UF milk was divided into three portions. pH of the first portion was adjusted to 6.4 using 1N HCL. The pH of the second portion was the normal pH of the UF retentate (6.7). The third portion was adjusted to pH 7.0 using 1N NaOH.

Each portion of adjusted pH retentate was divided into four parts. The first part was spray dried directly. The second part was heat treated at 65°C for 30 min, the third part was heat treated at 75°C for 28 sec while the forth part was heat treated at 85°C for 28 sec prior to spray drying. All powder samples were stored at 4°C until analysis for chemical composition, physical and functional properties.

#### **Results:**

Table 1 indicates the effect of pH values and heat treatments prior to spray drying on the chemical composition of skim milk retentate powder. The analysis of the powdered samples of the UF retentate for physical properties are on going, while the other functional properties will be completed at the end of next month.

We will be able to define the best combination of adjusted pH and heat treatment when finished with the statistical analysis of the obtained results, which forms about 80% of the first objective of the project. The remaining 20% of the first objective will be covered through the enzyme treatment in content with the best combination of adjusted pH and heat treatment. It will take about three months or until the end of December to complete this first objective.

#### **Publications:**

and, S.A. and C.L. Hansen. 1991. Whippability of Enzyme Treated Skim Milk  
tate Powder. Accepted for presentation at American Dairy Science Association  
Annual Meeting, August 12-15, 1991, Utah State University, Logan.

Two others are in preparation.

Table 1. Effect of pH values and heat treatments on the chemical composition of skimmilk retentate powder.

HT	Moisture		Protein		Lactose		Fat		Ash		T.A.		pH	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
pH 6.4														
NH	5.44	1.01	59.29	0.79	27.40	1.22	0.88	0.34	6.99	0.56	0.17	0.01	6.43	0.06
I	5.19	0.77	58.78	1.22	28.05	1.96	0.85	0.40	7.13	0.11	0.17	0.01	6.43	0.06
II	5.56	0.87	59.14	2.36	27.63	2.63	0.50	0.36	7.17	0.14	0.17	0.01	6.40	0.00
III	5.33	0.90	56.68	1.01	30.10	2.18	0.62	0.31	7.27	0.07	0.19	0.02	6.43	0.06
pH 6.7														
NH	4.00	0.77	60.15	0.38	27.22	0.83	1.39	0.74	7.24	0.20	0.13	0.01	6.68	0.03
I	4.91	1.05	58.85	2.65	28.25	3.06	0.89	0.62	7.10	0.11	0.14	0.01	6.73	0.06
II	5.43	1.73	57.90	3.04	28.14	3.00	1.38	0.89	7.15	0.25	0.14	0.00	6.67	0.03
III	5.61	0.64	57.35	0.96	29.15	0.68	0.53	0.15	7.36	0.00	0.15	0.01	6.70	0.00
pH 7.0														
NH	5.23	0.28	59.45	1.22	26.54	0.91	1.34	0.73	7.46	0.26	0.10	0.02	6.97	0.06
I	5.06	0.34	59.09	2.22	27.68	2.49	0.78	0.45	7.39	0.06	0.11	0.00	7.00	0.00
II	5.28	0.63	58.82	1.64	27.92	1.83	0.75	0.82	7.42	0.14	0.10	0.00	6.93	0.06
III	5.78	1.00	56.54	0.12	29.86	1.23	0.42	0.22	7.40	0.02	0.12	0.01	6.95	0.09

HT : Heat Treatment.

NH : No Heat Treatment.

I : Heat Treatment at 65°C.

II : Heat Treatment at 75°C.

III: Heat Treatment at 85°C.

**Project Title:** Membrane Fractionation of Immunoglobulins from Milk and Whey

**Personnel:** Paul A. Savello, Dept. of Nutrition and Food Sciences, Utah State University

Reyad Mahmoud, Dept of Nutrition and Food Sciences, Utah State University

**Funding:** Western Dairy Foods Research Center

**Objectives:**

1. Fractionate immunoglobulin protein components from skim milk and whey using sequential micro-, ultra-, and nano-filtration technologies;
2. Optimize the fractionation procedures and conditions to obtain the highest purity of desired protein component per separation stream;
3. Concentrate and freeze dry the protein fraction streams for laboratory analysis of fractions' purity by HPLC.

**Results:**

Whey and skim milk were subjected to separation by a wide range of separation modules. Spiral wound polysulfone membranes (2,000, 5,000, 15,000 and 40,000), mineral membranes (10,000 and 80,000) molecular weight cut-off, and ceramic membranes (0.05, and 0.2 micron) pore size were tested. Permeates of sweet whey and skim milk were analyzed using HPLC gel permeation column.

The analysis of permeate was used to indicate the molecular range cut-off of the membranes. Polysulfone membranes below 4,000 molecular weight cut-off prevented almost all the whey proteins permeation. The 40,000 molecular weight cut-off membrane permeate indicated a complete retention of immunoglobulins in the retentate and partial permeation of  $\alpha$ -LA and  $\beta$ -LG in the permeates of whey and skim milk. Permeates of skim milk and whey from ceramic membrane 0.2 micron allowed all the whey proteins to permeate including the immunoglobulins. However, the analysis of a ceramic membrane 0.05 micron permeates exhibited a slight permeation of  $\alpha$ -LA and  $\beta$ -LG. Permeate of mineral membranes indicated partial permeation of  $\alpha$ -LA and  $\beta$ -LG and retention of immunoglobulins.

filtration and concentration of retentates of skim milk and whey of the three membranes (40,000 cut-off polysulfone, 100,000 mineral, and 0.05 ceramic) will be conducted to achieve the third objective.

#### **Impact of Research:**

The extraction of immunoglobulins from whey would have a positive impact in adding value product.

# Microbiology of Starter Cultures

Effect of Proteolytic Enzymes on Transfection and Transformation  
of Streptococcus lactis Protoplasts†

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† This paper is published with the approval of the  
Director of the Utah Agricultural Experiment Station,  
Utah State University, Logan, UT, as Journal paper  
No. 3360

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# ABSTRACT

By using both chymotrypsin and mutanolysin to form protoplasts consistent transformation frequencies of  $10^4$  to  $10^5$  transformants and transfectants per  $\mu\text{g}$  of DNA were achieved. The procedure was used to transform protoplasts of Streptococcus cremoris CS224 at low frequency (5 transformants per  $\mu\text{g}$  of DNA).

A reliable and efficient procedure for introducing DNA into dairy starter cultures is essential for applying genetic engineering techniques to these industrially important bacteria. In 1982, polyethylene glycol (PEG) induced transformation (11) of Streptococcus lactis and transfection (8) of Streptococcus lactis subsp. diacetylactis protoplasts were described. Since then, several protoplast transformation procedures have been developed for different strains of Streptococcus lactis and low frequency transformation of Streptococcus cremoris has been reported (17). Mutanolysin (11, 13), lysozyme (14, 18, 22) or combinations of both mutanolysin and lysozyme (7) have been employed to form protoplasts for transformation. The efficiency of protoplast formation, regeneration, and transformation appear to be strain specific. Despite the many published procedures, transformation frequencies have been variable ranging from  $10^2$  to  $10^6$  transformants per  $\mu\text{g}$  DNA.

Transformation of protoplasts generated from lysozyme treatment has been inefficient with our strains of S. lactis (unpublished results). Protoplasts formed from mutanolysin treatment are effective for transformation but transformation frequencies may vary considerably depending on the particular commercial lot of mutanolysin (unpublished results). Commercial sources of this enzyme are known to contain different muralytic, proteolytic (19, Sigma Chem. Co. personal communication) and DNase activity (23). The proteolytic activity of different commercial lots of mutanolysin may vary from less than 0.01% to 0.04% of the lytic activity (Sigma Chemical Company, personal communication). This proteolytic activity is known to stimulate lytic activity of various enzymes (20) and it has been speculated that proteolytic enzymes enhance lytic activity by their clearing action against cell debris (26).

In this study the efficiency and repeatability of transformation and transfection could be improved by forming protoplasts with mutanolysin in conjunction with proteolytic enzymes. By optimizing various parameters, we developed a method which consistently results in  $10^4$  to  $10^5$  transformants and transfectants per  $\mu\text{g}$  of DNA when using protoplasts from S. lactis LM2301. We have also used this method to transform S. cremoris CS224 at low frequencies.

**Bacterial strains, bacteriophages, and plasmids.** S. lactis LM2301, a plasmid-cured derivative of S. lactis C2 (5), was used as a recipient for transformation and transfection experiments. S. cremoris CS224, received from Dr. William Sandine (Department of Microbiology, Oregon State University, Corvallis) contains 7 plasmids with masses of 57, 35.8, 27, 26, 20.5, 16.8, and 1.8 megadaltons (Mdal). Cultures were maintained by biweekly transfer at 30°C in M17 broth (21) containing glucose (M17G) or lactose (M17L). S. lactis JK301 (14), which contains pG8301 (2, 3) was used to isolate plasmid DNA for transformation experiments. Plasmid pG8301 is a 6.5 Mdal Streptococcus sanguis cloning vector coding for MLS (macrolide, lincosamide, streptogramin B) and chloramphenicol (Cm) resistance. S. lactis C2 lytic bacteriophage c2 (16) was propagated by infection of S. lactis C2 in M17 broth.

**DNA Isolation Procedures.** Bacteriophage was isolated by infecting S. lactis C2 with c2 bacteriophage and incubated at 30°C until complete lysis was observed (3 to 5 h). Bacteriophages were PEG concentrated by the method of Yamamoto and Alberts (25) and DNA was extracted and purified as previously described (8). Plasmid DNA was isolated by the method of Anderson and McKay (1). Plasmids used for transformation were further purified by cesium chloride-ethidium bromide equilibrium density gradient centrifugation (10).

Desalting was performed by repeated ultrafiltration in a Centricon 30 microconcentrator (Amicon division, W.R. Grace & Co., Danvers, Mass.). Plasmids and bacteriophage DNA were visualized by electrophoresing DNA samples through a 0.6% horizontal agarose gel in TB buffer (0.089 M Tris hydrochloride, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) for 4 h at 6 volts/cm. DNA concentration was determined spectrophotometrically (absorbance at 260 nm/absorbance at 280 nm).

**Protoplast formation.** The final procedure for formation of protoplasts of S. lactis LM2301 was performed as described previously (13) with modifications. A 1% inoculum of an active M17G broth culture was made into 30 ml of M17G broth prewarmed to 30°C. Cells were grown for 4 or 16 h overnight at 30°C ( $2.5 \times 10^9$  CFU/ml), centrifuged at  $7,600 \times g$  for 5 min, washed in cold (4°C) double deionized water, and suspended in 7.6 ml of 0.5 M sucrose in 0.01 M Tris hydrochloride, pH 7.0. Mutanolysin (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 25 U/ml followed immediately by addition of chymotrypsin (Boehringer Mannheim Biochemicals, Indianapolis, In.) to a 1.25 U/ml final concentration. The cell suspension (3 ml final volume) was incubated at 37°C for 15 min, centrifuged at  $2,000 \times g$  for 10 min, washed in 5 ml SMMB buffer (0.5 M sucrose, 0.02 M maleate, 0.02 M  $MgCl_2$ , 1% bovine serum albumin, pH 6.5), and resuspended in 1.0 ml of SMMB. Resuspension of protoplasts was performed in a Lab Line orbit environ-shaker (Lab-Line Instruments, Inc., Melrose Park, Il.) on a setting of 200 RPM at 30°C.

**Transformation.** Transformation experiments were performed as previously described (13) with modifications in plating methods. A one-tenth ml aliquot of an appropriate dilution of transformed cells was added to 5 ml of M17G top

agar (0.5% agar) containing 0.5 M sucrose as an osmotic stabilizer (SM17G), gently mixed, and overlayed on SM17G bottom agar (1.5% agar) containing 5  $\mu$ g Cm per ml. Later, cells were plated by the spread plate technique onto SM17G bottom agar supplemented with 5  $\mu$ g Cm per ml. Transformants were scored after 7 days of incubation at 30°C. Protoplast concentration was kept constant for each transformation trial by pooling protoplast suspensions before distributing them for transformation. SMMB buffer was filtered through a 0.45  $\mu$ m membrane filter before use while all other reagents and buffers were autoclaved and quickly cooled in a water bath. All transformation experiments were from two or more independent trials. No transformants were observed when cells were incubated with chymotrypsin alone or when DNase I (Sigma) was added to plasmid preparations before transformation.

**Transfection.** Transfection was performed essentially the same as transformation. Appropriate dilutions of the transfected protoplasts were made in SMMB buffer and plated by adding 0.1 ml of protoplasts to 3 ml of SM17G top agar (0.5% agar) containing 0.1 ml 1 M  $\text{CaCl}_2$ , and 0.2 ml indicator cells (S. lactis LM2301). PFU were counted after 24 h of incubation at 30°C. Treatment of bacteriophage DNA with DNase I inhibited transfection. No plaques appeared when bacteriophage DNA was not added and the DNA used for transfection was free of PFU in conventional phage assays.

Preliminary experiments indicated that chymotrypsin treatment of cells during protoplast formation with mutanolysin enhanced transformation and transfection of S. lactis LM2301 (Table 1). Parameters affecting transformation and conditions necessary for optimal transformation frequencies were then examined.

**Effect of chymotrypsin, pronase, and trypsin on transformation frequency.** Protoplasts of S. lactis were prepared for transformation by treating cells with various concentrations of mutanolysin and chymotrypsin and incubating for different time periods (Table 2). Incubation for 15 min increased transformation frequencies but only when 1.25 or 6.25 U/ml of chymotrypsin was used. After 30 min of incubation, only the lowest concentrations of the two enzymes increased transformation efficiency. Formation of protoplasts using 25 U/ml mutanolysin, 1.25 U/ml chymotrypsin, and an incubation time of 15 min were found to be optimal in further experiments (data not shown).

Pronase and trypsin were compared to chymotrypsin to assess the effect of different proteases on transformation frequency. Chymotrypsin ( $3.1 \times 10^4$  transformants per  $\mu\text{g}$  DNA) and trypsin ( $2.8 \times 10^4$  transformants per  $\mu\text{g}$  DNA) increased the transformation frequency approximately 100 fold than when mutanolysin was used alone ( $5.1 \times 10^2$  transformants per  $\mu\text{g}$  DNA), while pronase only increased transformation frequency to  $1.3 \times 10^3$  transformants per  $\mu\text{g}$  DNA.

**Effect of cell growth stage.** Transformation appears dependent on cell growth phase and on the extent to which cell wall material is removed from the cells (18). In order to examine the effect of cell growth stage, protoplasts were formed from 1, 2, 3, 4, or 5 h cultures of S. lactis. Cell densities were adjusted to approximately  $5 \times 10^9$  CFU/ml (1.2 optical density at 600 nm) for each time period prior to protoplast formation and transformation. Transformation was performed as described in materials and methods and plated by the soft agar overlay technique. Results in Fig. 1 show a sharp decrease in transformation frequency using protoplasts prepared

from 2 h cultures (optical density of 0.15 at 600 nm), followed by an increase in transformation frequency to  $2.0 \times 10^4$  transformants per  $\mu\text{g}$  DNA using protoplasts prepared from 4 h cultures (optical density of 1.2 at 600nm). Transformation frequencies using protoplasts prepared from 4 h cultures treated with mutanolysin only ( $5.7 \times 10^3$  transformants per  $\mu\text{g}$  DNA) were lower than when mutanolysin plus chymotrypsin were used.

Protoplasts were then formed from 2, 4, 6, and 16 h (overnight) cultures of *S. lactis*. Cell density was adjusted to 1.2 optical density at 600 nm for each time period prior to protoplast formation and transformation was performed as before except that transformed protoplasts were spread plated instead of overlaid. Results in Fig. 1 show that 16 h cultures (0 time) were transformed optimally (ca.  $7.0 \times 10^4$  transformants per  $\mu\text{g}$  DNA). Transformation frequencies were lower when 16 h cultures were treated with mutanolysin only ( $4.5 \times 10^3$  transformants per  $\mu\text{g}$  DNA). Transformation frequencies at 2 h showed greater variability ( $3.7 \times 10^4$  to  $2.8 \times 10^3$  transformants per  $\mu\text{g}$  DNA) when protoplasts were spread plated which is similar to the findings of Kondo and McKay (13).

Transfection of protoplasts prepared from 2, 4, 6, and 16 h cultures showed optimal frequencies when protoplasts were formed from 16 h ( $2.0 \times 10^5$  transfectants per  $\mu\text{g}$  DNA) and 4 h ( $1.0 \times 10^5$  transfectants per  $\mu\text{g}$  DNA) cultures. Transfection frequencies were lower when protoplasts were prepared from 2 h ( $4.7 \times 10^4$  transfectants per  $\mu\text{g}$  DNA) and 6 h ( $5.3 \times 10^4$  transfectants per  $\mu\text{g}$  DNA), but the decrease in frequencies at those time periods was not as pronounced as the decrease in transformation frequencies. Transfection frequencies using mutanolysin alone were lower for both 4 h ( $4.2 \times 10^3$  transfectants per  $\mu\text{g}$  DNA) and 16 h ( $3.0 \times 10^3$  transfectants per  $\mu\text{g}$  DNA)

cultures.

**Cell lysis.** The rate of production of osmotically fragile cells during treatment with mutanolysin and chymotrypsin was examined. Cultures were grown for 1, 2, 3, 4, or 16 h and resuspended in 0.01 M Tris hydrochloride (pH 7.0) without osmotic stabilizer. Cell lysis was followed by monitoring optical density over time. Fig. 2 shows that 2 h cultures are more susceptible to lysis than are 4 or 16 h cultures. When 4 h cultures were treated with both enzymes there was a greater and more rapid production of osmotically fragile cells compared to when 4 h cultures were treated with mutanolysin alone.

**Transformation of osmotically stable cells.** Transformation of osmotically stable cells, cells which have not formed protoplasts and thus are resistant to osmotic shock, was performed to determine the amount of cell wall removal necessary for DNA uptake. Osmotically stable cells were isolated after protoplast formation by dilution in sterile water and transformed as described in material and methods except that cells were plated on M17G containing 5  $\mu\text{g/ml}$  Cm. No transformation of osmotically stable cells was observed while  $5.0 \times 10^4$  transformants per  $\mu\text{g}$  of DNA were obtained using protoplasts. The fraction of protoplasts able to transform was only 0.002 % of viable protoplasts which results in a transformation efficiency of  $1.7 \times 10^{-5}$  transformants per viable protoplast.

**Effect of osmotic stabilizers and plating method.** Succinate has been used as an osmotic stabilizer in media used for regeneration of S. faecalis protoplasts (23) and may be effective in increasing transformation frequencies in S. lactis (L. L. McKay, personal communication). To test the effect of plating method and the influence of different osmotic stabilizers



transformants were either spread plated or overlayed onto M17G agar supplemented with either 0.5 M sucrose (SM17G) or 0.25 M succinate (SaM17G). Transformation frequencies of  $10^5$  transformants per  $\mu\text{g}$  of DNA could be achieved by overlaying or spread plating onto SaM17G. However, more consistent results were obtained when protoplasts were spread plated onto SM17G ( $2.5 \times 10^5$  transformants per  $\mu\text{g}$  DNA).

**Effect of PEG-DNA treatment time for induction of transformation.** The time of PEG-DNA treatment of protoplasts has been shown to affect transformation frequency. Polyethylene glycol treatment times of 2 min (18), 5 min (22), and 20 min (7, 13) have been reported for optimal transformation of different strains of S. lactis and a 10 min PEG-DNA treatment time is used for transfection of S. lactis subsp. diacetylactis (8). To examine the effect of PEG-DNA treatment time, protoplasts were treated with PEG and DNA for 2, 5, 10, and 20 min. We found maximal transformation frequencies were achieved after a 20 min PEG-DNA treatment time though shorter times could still be used. These results agree with those previously observed by Kondo and McKay (13).

**Transformation of S. cremoris.** The procedure described was used to transform S. cremoris to  $\text{Cm}^r$  with plasmid pGB301 at low frequencies (5 transformants per  $\mu\text{g}$  of DNA). Acquisition of pGB301 was suggested by the  $\text{Cm}^r$  phenotype and was confirmed by agarose gel electrophoresis of plasmid DNA isolated from transformants. All transformants (designated S. cremoris SW301) contained the normal complement of plasmids plus a newly acquired plasmid of approximately 6.5 Mdal which comigrated with pGB301 (Fig. 3). Therefore, it may be possible to develop high efficiency transformation of S. cremoris using pGB301 since it is able to replicate and express in th

strain.

It is unknown why proteolytic enzymes enhance transformation, but it appears that DNA uptake rather than regeneration of protoplasts is affected since regeneration-independent transfection frequencies are also increased. If proteases act by clearing cell debris (26), it is possible that observed increases in transformation frequencies are due to more efficient protoplast formation or that DNA is more accessible to protoplasts.

Proteases may also degrade cell wall and cell membrane proteins which inhibit transformation and transfection. Hurst and Stubbs (9) reported the appearance of holes in the cell wall of S. lactis which are blocked by trypsin sensitive conical protrusions. Removal of conical protrusions by proteases may open the holes and allow for better DNA uptake. Other cell wall and cell membrane proteins which bind DNA and make it unavailable for uptake by protoplasts, may be degraded by proteases. DNases secreted during protoplast formation may also decrease transformation and transfection frequencies (15). Using the DNase plate assay (Difco Laboratories, Detroit, Mich.), we observed extracellular DNase activity only after formation of protoplasts (unpublished results). It is possible that protease treatment during protoplast formation is able to degrade transformation inhibiting DNases.

Using lysozyme to form protoplasts, Simon et al. (18) found that optimal transformation was achieved from early-log phase (2 h) cultures. These differences may reflect the amount of cell wall removed during protoplast formation. The streptococcal cell wall is composed of polysaccharide and trypsin-sensitive mucopeptides which are present at greater amounts in stationary phase cultures (24). The susceptibility of streptococcal cells to

the action of either enzyme is dependent on the age of the culture prior to the enzyme treatment (4, 6). Also, mutanolysin has been shown to be more active in degrading cell walls of S. lactis (12). The weaker activity of lysozyme and the susceptibility of cell walls during early-log-phase may explain why 2 h cultures treated with lysozyme are transformed optimally, whereas optimal transformation using mutanolysin- and chymotrypsin-treated cells requires longer growth periods. Therefore, it appears that there is a critical amount of cell wall which must be removed for optimal transformation. Simon et al. (18) have demonstrated that optimal transformation frequencies are obtained when there is limited cell wall digestion. However, we have found that there must be enough cell wall removed to render cells osmotically sensitive.

The transformation procedure reported here has eliminated much of the variability that we have observed in transformation frequencies. The proteolytic activity of commercial sources of mutanolysin may be a significant factor affecting the variability of transformation frequencies. By careful reagent preparation and handling of protoplasts, and formation of protoplasts using a combination of mutanolysin and chymotrypsin, transformation frequencies have been consistent. We also have not had to readjust enzyme concentrations and treatment times with each new lot of mutanolysin. However, the procedure is still strain dependent and conditions for transformation of new strains may have to be optimized.

This project was funded by the National Dairy Promotion and Research Board and is administered in cooperation with the Dairy Research Foundation.

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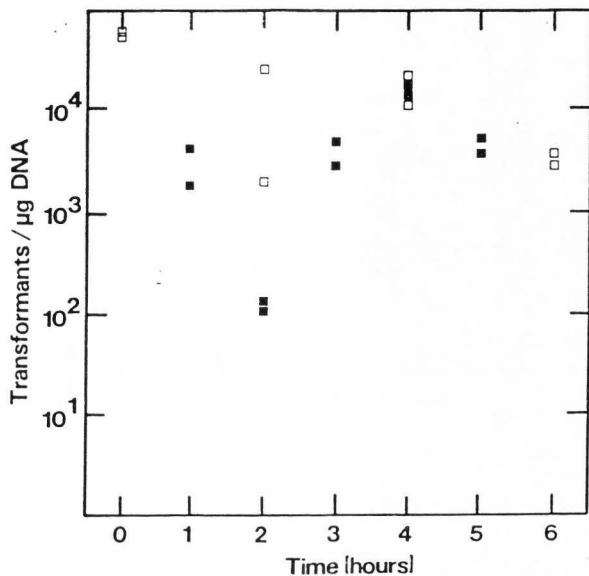


FIG. 1. Effect of growth stage of cultures used to form protoplasts on transformation frequency. Protoplasts of *S. lactis* LM2301 were prepared from cultures taken after 1, 2, 3, 4, 5, 6 and 16 h (0 time) of incubation. Protoplasts were transformed as described in material and methods and either plated by the overlay technique (■) or by spread plating (□).



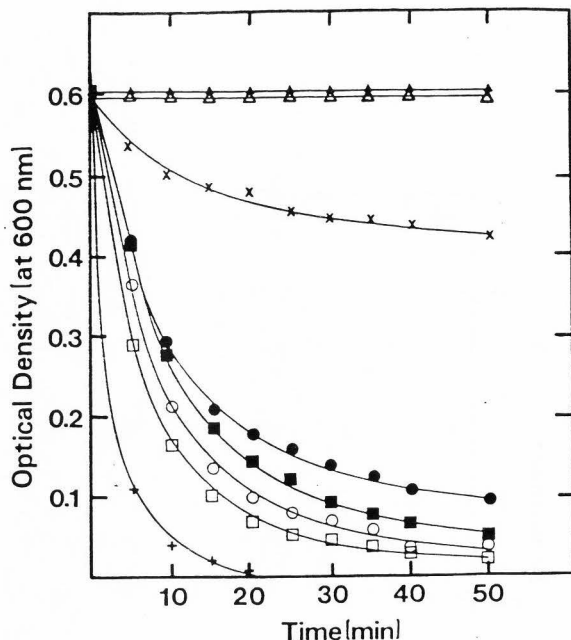


FIG. 2. Time course for the production of osmotically fragile cells of *S. lactis* LM2301. Cells were incubated for various time periods and resuspended in hypotonic buffer. Mutanolysin and chymotrypsin were added and optical density at 600 nm was monitored. Controls;  $\Delta$ , 4 h cultures treated with chymotrypsin only;  $\blacktriangle$ , 4 h cultures with no enzyme treatment. Symbols:  $\blacksquare$ , 1 h of growth treatment with both enzymes; +, 2 h growth treatment with both enzymes;  $\square$ , 3 h growth treatment with both enzymes;  $\circ$ , 4 h growth treatment with both enzymes;  $\bullet$ , 4 h growth treatment with mutanolysin only; x, 16 h growth treatment with both enzymes.

FIG. 3. Agarose gel electrophoresis of plasmid DNA of parental and transformed strains. Lane A, E. coli V517 reference plasmids. Lane B, pGB301. Lane C, S. cremoris CS224 parental strain used as a recipient for transformation. Lane D, S. cremoris SW301, a Cm<sup>r</sup> transformant of S. cremoris CS224 containing pGB301.

Table 1. Transformation and transfection of mutanolysin and chymotrypsin treated cells of S. lactis LM2301.<sup>a</sup>

Mutanolysin	Chymotrypsin	Transfectants	Transformants
U/ml	U/ml	per $\mu$ g DNA	per $\mu$ g DNA
25	0	$3.1 \times 10^2$	$2.9 \times 10^1$
25	1.25	$4.6 \times 10^3$	$2.3 \times 10^2$
25	6.25	$1.3 \times 10^3$	$2.3 \times 10^2$
25	12.50	$1.9 \times 10^3$	$9.8 \times 10^1$
25	25.00	$9.3 \times 10^2$	$1.1 \times 10^2$

<sup>a</sup>S. lactis LM2301 cells were grown for 3 h (O.D. at 600 nm of 0.65), and treated with mutanolysin for 30 min, followed by chymotrypsin treatment for 15 min. Conditions for transformation include using 1  $\mu$ g of pGB301 DNA, a 20 min PEG treatment time, and selection of Cm<sup>r</sup> transformants by the overlay technique.

Table 2. Effect of mutanolysin and chymotrypsin concentrations and time of incubation on transformation frequencies of S.

lactis LM2301.<sup>a</sup>

<u>Enzyme Concentration (U/ml)</u>		<u>Treatment Time (min)</u>	
mutanolysin	chymotrypsin	15	30
		<u>(CFU/<math>\mu</math>g DNA)</u>	
25.0	0	$6.2 \times 10^2$	$3.9 \times 10^3$
12.5	1.25	$1.2 \times 10^4$	$1.2 \times 10^4$
12.5	6.25	$3.8 \times 10^3$	$2.0 \times 10^3$
12.5	12.5	$9.0 \times 10^2$	$3.6 \times 10^2$
25.0	1.25	$1.1 \times 10^4$	$8.4 \times 10^3$
25.0	6.25	$5.2 \times 10^3$	$1.5 \times 10^2$
25.0	12.5	$3.5 \times 10^2$	$2.9 \times 10^2$
50.0	1.25	$1.2 \times 10^3$	$1.9 \times 10^2$
50.0	6.25	$1.0 \times 10^3$	$1.7 \times 10^2$
50.0	12.5	$1.4 \times 10^2$	$3.6 \times 10^2$

<sup>a</sup> Protoplasts were formed from 3 h cultures (O.D. at 600 nm of 0.65) by adding different concentrations of mutanolysin plus chymotrypsin and incubating for either 15 or 30 minutes. One  $\mu$ g of pGB301 was used and transformed protoplasts were plated using soft agar overlays.

## GENETIC ANALYSIS OF PROTEINASE NEGATIVE LACTIC CULTURES

Craig Oberg and Gary Richardson

In an effort to characterize a number of proteinase positive cultures (Prt<sup>+</sup>) and their proteinase negative variants (Prt<sup>-</sup>), plasmid profiles of the individual strains were obtained. The plasmid complement of the strains varied greatly with differences appearing between the parental cultures and their proteinase negative derivatives. Several proteinase negative variants appeared to have lost a plasmid when compared to their parental cultures. These cultures were Streptococcus cremoris UC 318, UC 45, UC 169, and UC 310. Plasmid DNA from these was purified by CsCl equilibrium centrifugation and analyzed. No specific plasmid could be correlated to the loss of proteolytic activity in these proteinase negative variants although there was considerable variation.

Plasmid curing studies were then initiated by taking the proteinase positive parental strains and subjecting them to various treatments in order to remove plasmids. This should result in the generation of new proteinase negative variants. By determining which plasmid was lost in these new proteinase negative mutants, we could find where the proteinase genes resided in the parental strain. Three strategies were employed, screening for the spontaneous loss of proteolytic activity, protoplast curing, and the use of mutagenic curing agents. Spontaneous mutants of UC 169 were isolated from buffered milk agar plates. Ten isolates were eventually found that were proteinase negative. Although there were some detectable changes in the plasmid profiles, the loss of no single plasmid in all of the isolates could be shown.

Protoplasting, the removal and regeneration of the cell wall, was used in an attempt to cure out the proteinase plasmid in S. cremoris UC 45 and UC 310. Forty colonies of each were isolated from the regeneration plates. These

isolates were tested for the loss of proteolytic activity using the four tube coagulation test (cultures incubated in four tubes, one with 10% NFDM, one with 10% NFDM + .25% CAA, one with 10% NFDM + 1% glucose, and the last with 10% NFDM + .25% CAA + 1% glucose). Most of the UC 45 isolates were proteinase negative and no UC 310 proteinase negatives were found, although three Lac<sup>-</sup> variants were isolated. Promising isolates were retested with the four tube coagulation test. Plasmid profiles were run on all of the UC 45 Prt<sup>-</sup> isolates but the loss of no single plasmid in all isolates was observed.

Protoplast plasmid curing was then used with strains S. cremoris UC 73 and UC 320. Nineteen isolates of UC 73 and 152 isolates of UC 320 were tested using the four tube coagulation test. All promising isolates were retested. Five proteinase negative variants of UC 320 were found, and two proteinase negative UC 73 isolates were obtained. Agarose gel examination of the plasmid profiles showed that once again there were changes in the plasmid profile but no single plasmid could be shown to be lost in all the proteinase negative isolates of either strain.

Five proteinase negative mutants of UC 320 were obtained by acriflavin treatment from 50 isolates initially isolated and screened. There were changes in the plasmid profile but the loss of no single plasmid could be associated with the loss of proteolytic activity in the proteinase negative variants. It was thought that these proteinase negative variants may have been generated due to point mutations so this technique was not used again.

Following the observation in Jeff Kondo's laboratory that S. cremoris UC 320 could conjugally transfer the ability to use lactose to a plasmid cured strain, S. lactis LM2301, attempts were then made to transfer proteinase genes conjugally. If this occurred then the plasmid from the proteinase positive S. cremoris strains might appear in the recipient strain LM2301. Eight strains of S. cremoris were tested to see if they would conjugally transfer genetic material to S. lactis LM2301, a strain containing no plasmids. This strain is

Lac<sup>-</sup> and Prt<sup>-</sup>. Transconjugants were isolated on FSDA II agar, which allows for selection based on lactose utilization and proteinase activity. S. cremoris UC 73 and UC 169 matings produced what appeared to be transconjugants, but upon plasmid profiling it appeared that the streptomycin resistant genes had transferred from LM2301 to UC 73 instead of proteinase genes transferring from UC 73 to LM2301. The conjugal matings of UC 320 with LM2301 produced a wide variety of transconjugants. These were plated out on FSDA II agar and the colonies that appeared to be Lac<sup>+</sup> and Prt<sup>+</sup> were tested using the four tube coagulation test. Promising isolates were tested two more times to confirm their phenotype. Three isolates (TL7, TL23A, and TL24) were found to be Lac<sup>+</sup> and Prt<sup>+</sup>. HPLC analysis of the proteolytic activity of these compared to a Lac<sup>+</sup> Prt<sup>-</sup> tranconjugant and to LM2301 showed that these transconjugants definately were proteinase positive. This confirmed that there had been a transfer of the proteinase genes to LM2301. Plasmid profiles of these Prt<sup>+</sup> transconjugants revealed no observable plasmids. Acriflavin treatment of the transconjugants was used to try and cure out the Lac genes while still retaining the Prt genes. Fifty Lac<sup>-</sup> isolates were tested and all turned out to be Prt<sup>-</sup>. High temperature curing (growing the cultures at 37 or 39° C to stress the cells into potentially losing plasmids) was then tried using transconjugant TL 23A. Thirty four Lac<sup>-</sup> isolates were tested with two showing possible retention of proteinase activity (T21 and T35). Plasmid analysis of these two derivatives showed no observable plasmid bands.

These results indicate that the proteinase genes may have been incorporated into the chromosome of LM2301. Further work on these strains is presently being done. This same phenomenon was recently noted by Steele and McKay where they observed that an extremely large plasmid of approximately 88 mdsal was containing the proteinase genes was transferred. We are now presently using a slight modification in the plasmid isolation method to try and determine if a very large plasmid might be present in our proteinase

postive transconjugants. Hybridization experiments using an established proteinase plasmid from S. cremoris WG2 to probe the chromosome of the LM2301 transconjugants and look for integrated proteinase genes from UC 320 are being started.

The proteinase system in lactic streptococci is very complicated consisting of proteinase and peptidase genes and transport mechanisms. It appears that some of these are on transposable elements, particularly from the observations with the UC 320 transconjugants. As will be mentioned in the HPLC paper there are a wide variety of proteinase negative culture types and we are trying to better understand the proteinase system in these organisms.



High Performance Liquid Chromatography Analysis for Cheese Yield  
with Proteinase Negative strains of Streptococcus cremoris

C. J. Oberg, F. A. Khayat, and G. H. Richardson

Many tests have been used to analyze the proteolytic characteristics of lactic cultures. These tests can distinguish between proteinase positive and negative strains but are limited in differentiating among proteinase negative variants. High performance liquid chromatography analysis has been used to separate bitter peptides from cheese (2), examine proteolysis in raw milk (8), detect harmful amines in Cheddar cheese (3), and follow proteolysis in cheese curing (7). We applied HPLC technology to characterize the proteolysis of proteinase positive and negative strains of Streptococcus cremoris.

Thirteen pairs of proteinase positive and negative cultures were obtained from our culture bank. Samples were prepared by inoculating 0.1 mL of an 18 hour culture into 10 mL of M-17 lactose broth and incubating the culture for 12 hours at 30° C (absorbance of greater than 1.5). The culture was transferred to a sterile centrifuge tube and spun for 10 minutes. The pellet was washed in 10 mL of sterile distilled water and recentrifuged. Two mL of a 2.5% casein solution was added to the tube and the pellet resuspended. The 2.5% casein solution was prepared by mixing 0.5 g of rennet-treated casein powder with 19.5 mL sterile distilled water. The casein culture mixture was incubated at 30° C for 24 hours. After the incubation, 1 mL was transferred to a 1.5 mL microfuge tube and spun in a microfuge for one minute to facilitate filtering by pelleting out the cells. The supernatant was withdrawn into a 5 cc syringe and filtered through a Gelman ARCO LC 13 0.45  $\mu$ m filter. Samples were refrigerated and analyzed the same day that they were prepared.

Samples were analyzed by reverse phase high performance liquid chromatography using a Beckman Model 420 controller, a mixer, a variable wavelength detector, and an integrator. A Hamilton PRP-1 column with a

Hamilton cartridge guard column was used (6). This column was more resistant to the low pH of the solvent system than other columns evaluated. The extremely low pH (between 1 and 2) of the solvent system caused the packing material to slowly degrade and be eluted in other columns used. This was significantly reduced with the Hamilton column. The use of less trifluoroacetic acid, from 0.1% to 0.05 or .025%, in the solvents may also help. Careful monitoring of the column is required to insure repeatability. Samples were eluted using a solvent gradient (1). Solvent A was HPLC grade distilled water containing 0.1% HPLC grade trifluoroacetic acid (TFA) (pH of 2.2) and solvent B was 0.1% trifluoroacetic acid in 90% HPLC grade acetonitrile (pH of 1.2). Solvents were pre-filtered. During the 35 minute run the percent of solvent B was increased from 0 to 40%. The solvent flow rate was 1 ml per minute. An attenuation of 16 and a chart speed of 1 cm per minute were used. The detector was set at 220 nm and the sample loop contained 50 ul of filtered substrate. All samples were run at ambient temperature.

A fairly flat baseline resulted when the 2.5% casein substrate was analyzed (Figure 1). This casein control had received the same incubation as the other samples to see if autolytic peaks were formed during the 30°C incubation period. This was not the case although a soluble casein fraction peak was found to elute at approximately 34 minutes. This peak was found to decrease in area when cultures were incubated with the casein substrate. A small rise was noted at the 7 minute mark which increased with column use. One possibility was that the packing of the column was eluting with prolonged use. This problem may be able to be corrected by changing the guard column more often, increasing the wash time for the column, or raising the pH of the solvents. This was not found to be significant in obtaining useable results, but should be monitored closely for problems that could arise.

Incubated cells were analyzed alone to see if peaks were breakdown

products of casein or just peptides secreted from the cells. Pellets of S. cremoris UC 85 Prt<sup>+</sup> were resuspended in 2 ml of sterile distilled water without casein, incubated, and prepared as previously mentioned (Figure 2). HPLC runs of these samples showed a flat base line with no peaks. This confirmed that no competing peaks were generated from the pelleted cultures.

Standards containing known peptides were analyzed. Bradykinin, a peptide composed of nine amino acids, and the peptide angiotensin II, composed of eight amino acids were each run at a concentration of 0.025 mg per ml. The standards were prepared by suspending the peptide in sterile distilled water and filtering (Figure 3). The angiotensin II peak appeared at 29.2 minutes and the bradykinin peak appeared at 27.8 minutes. The use of these standards made it possible to quantitate peak areas. Running known peptide standards at a known concentration each day was a good check of the column and solvents.

Proteinase positive cultures of S. cremoris and their proteinase negative variants were prepared as previously described. Culture pairs were grown and incubated with the casein at the same time and run through the HPLC on the same day to minimize variability. Numerous peaks were produced with the cultured casein samples. It was decided that the profiles of four major peaks would be compared between samples. They appeared at approximately 9, 13, 19, and 24 minutes. The 34 minute peak found in the casein control was also monitored. The repeatability of peak areas was not as good as expected for the same cultures that were grown and prepared on different days. However ratios of peak areas between the proteinase positive culture and its proteinase negative variant did remain consistent for samples prepared on different days. The variability between runs may be minimized by adjusting the number of cells to a particular OD prior to pelleting.

As expected most of the proteinase positive cultures produced larger peak areas when compared with their proteinase negative counterparts. S. cremoris

UC 320 was much more proteolytic than its proteinase negative variant (Figure 4). The average peak area decrease for the four peaks on three trials was 44% (Figure 6). S. cremoris UC 310 also showed this pattern (Figure 7). The average peak area decreased 43% for the proteinase negative culture from the proteinase positive culture. These strains have been associated with the largest yield increases in casein, cottage, and Cheddar production (5,13,14). Of the other cultures tested UC 171 and UC 85 were also found to have a less proteolytic proteinase negative variant. UC 85 showed the greatest difference between the two proteinase types indicating its potential in yield increases in cheese manufacture.

In four of the culture pairs tested it was found that the peak areas were greater for the proteinase negative variant than for the proteinase positive parent. In five trials with UC 73 the proteinase negative variant produced peak areas that were 45% greater than those of the proteinase positive culture (Figures 5 and 8). This was also observed with S. cremoris UC 97 and UC 63 where the peak areas for the proteinase negative variant were greater than the proteinase positive parent (Figure 9).

Yield studies in the past comparing proteinase positive and negative variants have found that increases in yield is strain dependant (10,12). Yield data for a number of the strains examined correlates to the HPLC observations of proteolysis variation between the proteinase positive and proteinase negative variants. Heap and Richardson (5) found a 5.6% yield increase, based on casein yield, when the proteinase negative variant of S. cremoris UC 310 was compared to its proteinase positive parent in reconstituted skim milk (Figure 10). They also observed that S. cremoris UC 320 proteinase negative exhibited a yield increase over the proteinase positive strain. Stoddard and Richardson (13) found a 2.26% increase in yield for the proteinase negative variant of S. cremoris UC 310 over its proteinase positive counterpart in cottage cheese

manufacture (Figure 11). They observed no increase in yield for the proteinase negative strains of either S. cremoris UC 97 or UC 73. This would be predicted from the peak areas comparison for these two cultures since the proteinase negative strains had larger peak areas. Ekart, O'Leary, and Hicks (4) reported last year on yield increases associated with the use of proteinase negative cultures in cottage cheese. Winkel and Richardson (14) in simulated Cheddar curd manufacture experiments showed a statistically significant retention of nitrogen in the curd manufactured with S. cremoris UC 310 Prt<sup>-</sup> over the Prt<sup>+</sup> parent. They also noted a slight increase in yield for strains UC 318 and UC 320. Previously we reported that the proteinase negative variant of S. cremoris UC 73 produced no increase in yield over a commercial proteinase positive culture blend in the manufacture of Cheddar cheese (9). These yield comparisons support the observations made with the HPLC analysis for each culture. These results also may explain why an increase in soluble nitrogen was noted in cheese manufactured with the proteinase negative culture UC 73 during a cheese curing study (9).

In conclusion, the HPLC analysis of casein hydrolysates and the yield studies show that each proteinase negative variant should be analyzed before it is used to improve yield in the plant. Although proteinase negative cultures have long been identified based only upon their ability to coagulate milk slowly this does not give an accurate assessment of their proteolytic activity. Our studies suggest that some strains produce peptides that cannot be used by the cells for growth in milk. We also learned why some proteinase negative cultures cannot affect yield increases. Most proteinase negative cultures retain high peptidase activities and should be helpful in the reduction of bitterness problems. Even though some strains may be slow coagulators they might not increase the retention of casein in the cheese curd. This lack of a yield increase may be associated with increased peptidase activity or only the

loss of the transport mechanism, but not the loss of the proteolytic enzymes in a proteinase negative culture. The HPLC test shows potential for comparing newly derived proteinase negative variants with their proteinase positive parents to see if yield advantages will be available. It does not appear to be a reliable way to individually fingerprint cultures since it has been difficult to obtain an exact reproduction of the peak areas every time. We are presently analyzing the amino acid profiles of casein hydrolyzates to further differentiate and characterize cultures.

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Figure 1.



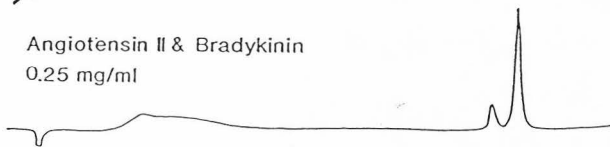
Figure 2.

UC 85+  
in Distilled water



Figure 3.

Angiotensin II & Bradykinin  
0.25 mg/ml



Angiotensin II  
.025 mg/ml

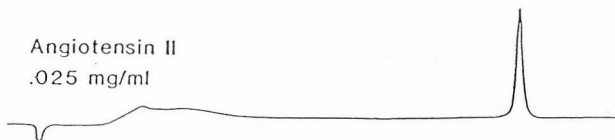




Figure 4.

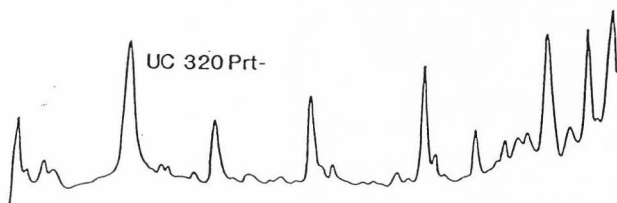
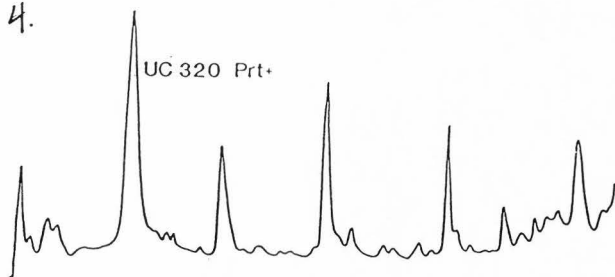


Figure 5.

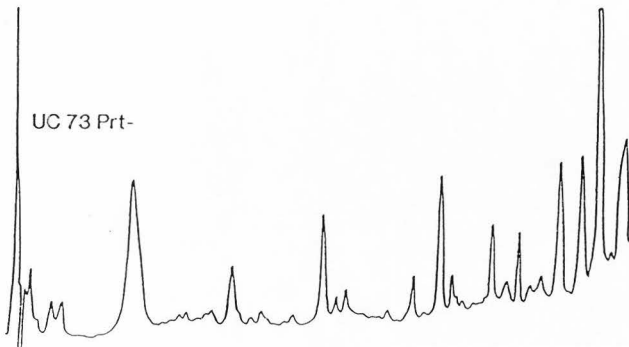
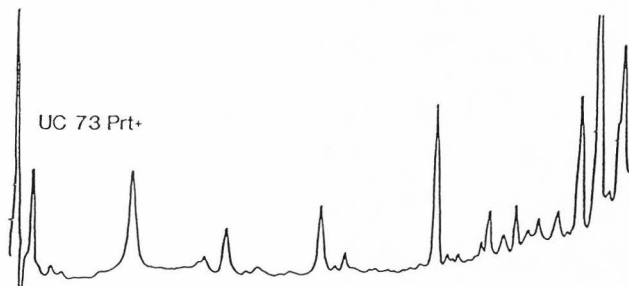


Figure 6.

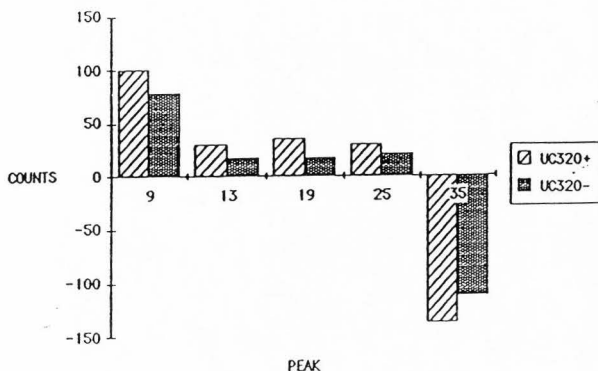


Figure 7.

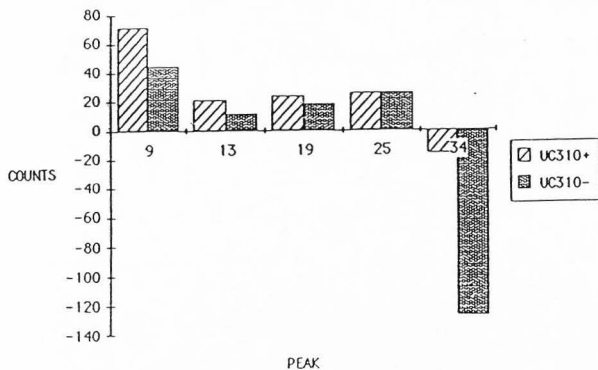


Figure 8.

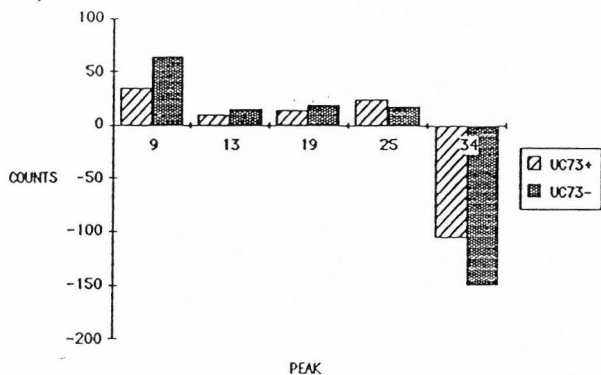


Figure 9.

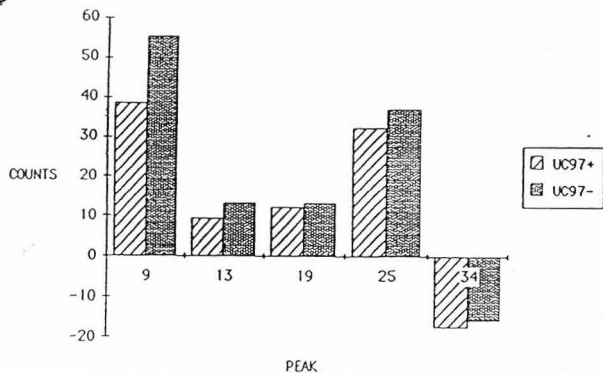


Figure 10.

Casein yields in RSM containing 0.1% yeast extract. DA, direct acid precipitation; +, fast-coagulating isolate; -, slow-coagulating isolate.

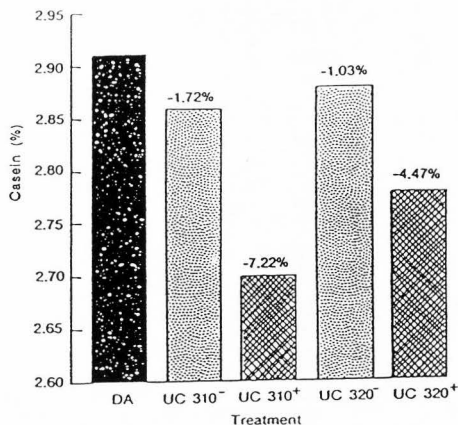
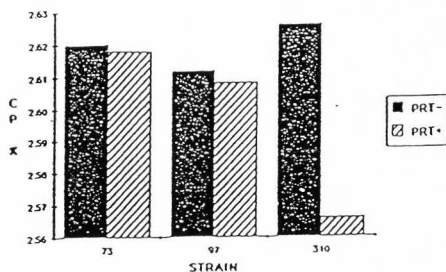


Figure 11.



Yield of casein protein (CP) resulting from the activity of proteinase-positive (Prt<sup>+</sup>) and proteinase-negative (Prt<sup>-</sup>) strains of UC73, 97, and 310 in reconstituted nonfat dry milk for cottage cheese.

WESTERN STATES DAIRY FOODS RESEARCH CENTER  
(Utah State, Oregon State and Brigham Young Universities)

Director of Business Affairs \_\_\_\_\_ Date \_\_\_\_\_  
Oregon State University

## Justification:

Improved Method for Quality Monitoring— Heat resistant (sporeforming) psychrotrophic bacteria produce proteolytic enzymes that are responsible for causing marked deterioration of milk quality. Various degrees of protein denaturation occur (sweet curdle and bitter off-flavor). The microbiological procedures for isolating, enumerating and confirming the presence of thermophilic psychrotrophs is laborious, time consuming and does not directly provide information about potential proteolytic activity. Currently, there is no available practical method for dairy processors to rapidly determine residual microbial proteases in heated milk. Development and adoption of a simple, sensitive and rapid assay for relatively low concentrations of microbial protease in raw milk could suffice to provide early awareness or warning about problem milk supplies. Such a method should be both qualitative and quantitative.

Impact on Increased Dairy Product Acceptance and Sales — The quantitation of microbial proteases in milk supplies is important from the standpoint of shelf-life and palatability of fluid milk and cream products. Improved palatability of milk and cream products as the sell-by-date (and one week beyond) is reached could serve to enhance consumer confidence in market milk and, hence, help reduce the gradual year-by-year reduction in fluid milk and cream sales. There may be no other more important single factor for limiting fluid milk/cream sales than "non-performance" of the product at the time of consumer expectation. Furthermore, undesirable microbial proteases in cheese milk serve to develop reduced flavor qualities in Cheddar and other types of cheese (primarily unclean and/or bitter off-flavors). It is also recognized that undesirable proteases can adversely affect the yield potential of given cheese milks.

In order for milk and cheese processors to minimize the detrimental effects of microbial proteases in milk supplies, there is an industry need for a more rapid and sensitive method for assessing these potential problems. The current method of monitoring milk supplies for thermophilic psychrotrophs entails an indirect microbiological procedure, which requires at least 10 to 12 days to complete. The availability of a more direct and rapid protease assay of milk (approximately 48 hours) would serve to identify and limit or eliminate problem milk suppliers.

Research and quality assurance efforts in improvement of milk product shelf-life, sensory qualities and enhancement of cheese yields, serve to increase consumer acceptance of all affected dairy products. Optimization of cheese yield from available milk supplies is an important factor in helping keep natural cheese competitive with other food alternatives for consumers.

Since it is currently impossible to destroy microbial proteases once they are formed in raw milk supplies, the dairy industry urgently needs a practical tool (test procedure) to monitor the relative presence or absence of this critical form of microflora. However, once the presence of this unwanted microflora and their associated deteriorative proteases is clearly demonstrated, a mere focus and application of basic farm sanitation practices can usually eliminate this critical problem. It must be emphasized that rapid, sensitive and positive identification of the problem microflora (and/or their related) enzymes is pivotal to resolving this emerging dairy industry problem.

Potential Pathogenicity of Bacillus sp. — An additional fact that should be reckoned with is that several of the Bacillus sp. (B. cereus and B. licheniformis, and possibly other species) are considered to be pathogenic. This unfortunate fact adds additional impetus to the relative importance of tracking the frequency of occurrence for thermophilic (sporeforming) psychrotrophs in raw milk supplies.

Table 1 shows the frequency at which various investigators have reported the occurrence of heat resistant psychrotrophs in raw milk supplies. In each instance, the procedure involved heat treatment of raw milk (producer) samples at 176°F (80°C) for 10 minutes, followed by either: (a) plating samples and incubating for 7-10 days at 45°F (7.2°C) or (b) incubating heated milk samples for 7-10 days, followed by plating for bacterial enumeration.

In a recent study conducted by Bodyfelt of Grade A raw milk in Oregon, 25% of 559 samples indicated "objectionable levels" of heat resistant psychrotrophic bacteria. A recent survey of pasteurized milk and cream products from Oregon and Washington processors indicated that 44% of 64 commercially pasteurized products demonstrated either coagulation ("sweet-curdle") and/or objectionable bitter off-flavor (Table 2).

TABLE 1. SUMMARY OF OBSERVATIONS FROM VARIOUS STUDIES OF HEAT RESISTANT PSYCHROTROPHS IN MILK SUPPLIES

Year	Location	No. of Samples	Isolated Heat Resist. Psychrotrophs	Investigator	Ref.
1962	Ohio	197	0%	Martin et al.	17
1971	Alabama	18	(83%)	Ching & Cannon	6
1971	California	97	30%	Shehata & Collins	24
1974	Tennessee	54	28%	Overcast & Atmaram	21
1976	Ohio	109	28%	Mikolajcik & Simon	19
1977	Oklahoma	227	59%	Washam et al.	29
1978	Ohio	13	100%	Mikolajcik	18
1982	Scotland		27%	Johnston & Bruce	13
1984	India	51	65%	Sharma et al.	22
1986	Oregon	559	25%	Bodyfelt	30
1987	Oregon	67	75%	Meer & Bodyfelt	31

TABLE 2. OBSERVATIONS OF COMMERCIALY PASTEURIZED MILK SAMPLES FOR DEVELOPMENT OF SWEET-CURDLE OR BITTER OFF-FLAVOR WITH RESPECT TO THE "SELL-BY DATE"

No. Samples	Fluid Product	When Product Became Unpalatable					
		Prior to "date" 0 to 5 days		After "date" 1 to 6 days		After "date" ≥ 7 days	
		No.	%	No.	%	No.	%
16	Skimmilk	8	50	3	19	5	31
16	2% Lowfat	4	25	7	44	5	31
16	Homogenized	5	31	7	44	4	25
16	Half-&-Half	11	69	4	25	1	6
64	All Samples	28	44%	21	33%	15	23%

(Oregon and Washington state derived samples. Bodyfelt (30)).

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## Objectives

This research project will:

1. Attempt to develop an economical, simple and rapid (practical) assay for microbial protease activity in heat treated milk samples. The "target" proteolytic enzymes are those specifically produced by heat resistant psychrotrophic bacteria, derived from the steps of harvesting, storing and transporting raw milk prior to processing.
2. To determine the applicability of prepared k-casein gels (in hematocrit tubes) for both qualitative and quantitative activity of protease enzymes of isolated, characterized thermotolerant psychrotrophs from milk sources.
3. To demonstrate that the extent of casein denaturation in hematocrit tubes is proportional to the overall protease activity of the milk samples evaluated.
4. To simultaneously assess microbial lipase activity of the thermotolerant microflora to more fully assess the potential for overall quality deterioration due to "heat resistant" enzyme systems.
5. To compare and evaluate the effectiveness of the casein-gel denaturation method with other more conventional procedures for determination of milk proteolysis (Hull, Keay and Wildi; Church, et al., etc.).

## Procedures

The research undertaken here proposes to develop a rapid, sensitive and convenient linear diffusion test that is capable of quantitating the relative protease activity of heat treated samples of raw milk obtained from varied conditions of farm sanitation.

Numerous procedures have been employed in the past to detect the extent of proteolysis in milk and milk products. The most widely practiced procedure has been the method developed by Hull (13) in 1947. Unfortunately, this method lacks sufficient sensitivity and can be awkward to use when numerous samples are involved. Rather complicated assay methods have employed carbon-14 (methylated) casein or radioactively labelled casein. Other approaches, with increased sensitivity, have involved direct determination of the end products of proteolysis through use of chemical reagents that react specifically with  $\alpha$ -amino groups (5). More recently, Church et al. (6) developed an assay for milk proteolysis based on the reaction of O-phthalaldehyde and  $\beta$ -mercaptoethanol with primary amines.

## Project Work Plan:

### General Approach -

1. Collect appropriate samples of raw milk from herd milks that represent a range of sanitary conditions for milk production. Transport and store samples at  $\leq 4^{\circ}\text{C}$ . Undertake all analyses for protease activity within 30 hours of collection.
2. Assess general microbial characteristics of raw milk samples with the aerobic Standard Plate Count (SPC) and Preliminary Incubation (PI) counts (sample incubation at  $12.8^{\circ}\text{C}$  for 18 hours followed by conduct of SPC).

3. Heat treat aliquots of raw milk samples in screw cap tubes in a water bath at 80°C for 12 minutes and immediately cool in an ice bath to approx. 20°C.
4. Conduct SPC on cooled heat treated milk samples (surviving microflora are considered to be heat resistant).
5. Perform assay(s) for residual protease on heat treated milk samples.
6. Store aliquots of heat treated milk samples for 7-10 days at 7.2°C.
7. Perform SPC on stored milk samples (any detected microflora are considered to be heat resistant psychrotrophs).
8. Perform "second set" of assays for microbial protease activity following the 7-10 days refrigerated storage period.
9. Sensory observations conducted on stored milk samples (note any development of gelation ["sweet curdle"], milkfat destabilization, formation of objectionable odor ("unclean" or "proteolytic"), atypical appearance (gel formation, graininess, wheying off or heavy viscosity) and/or development of a distinct bitter aftertaste).
10. Attempt to demonstrate relationships between microbial analyses, protease activity, shelf-life characteristics and sensory evaluation of milk samples.

Specific approach for measurement of protease activity

1. The measurement of protease activity in milk samples will be based on the method employed by Holmes et al. (12) and Thunell et al. (16). This approach involves the preparation of kappa casein-agar gels which are placed in sedimentation/hematocrit tubes, then sealed with paraffin wax and stored at 2°C until use.
2. Milk samples of various age and subjected to various preliminary incubation conditions (to enhance potential protease activity of surviving microflora) will be treated as follows:
  - a. One ml of H<sub>2</sub>O and 10 ml of 0.75 N trichloro-acetic acid (TCA) added to treated 5 ml milk samples and vortexed (6).
  - b. After 10 minutes, the above solution will be filtered through #2 filter paper and the subsequent filtrate frozen at -60°C until assayed by the methods of Holmes et al. (12) and Church et al. (6). The latter analysis will be employed as the control procedure for measurement of the extent of proteolysis in this research.
  - c. Standard curves of known proteolytic enzymes of determined concentrations will be prepared, using a plot of the logarithms of known concentrations of the enzyme against the diffusion distance of precipitated kappa-casein (white band).
  - d. As appropriate, various microbiological and biochemical tests for differentiating and characterizing heat resistant psychrotrophic bacteria will be applied to isolated microorganisms (8)(see Table 3).
  - e. Filtrates from various milk samples will be added to the diffusion substrate and efforts made to compare diffusion distances with the appropriate standard curves for known enzyme concentration.
  - f. As necessary, conduct analyses of milk samples inoculated with known heat resistant psychrotrophs (OSU isolates) to study and characterize the protease activity of typical microflora of this type and correlate to sensory and proteolysis observations.

TABLE 3. BIOCHEMICAL AND MICROBIOLOGICAL PROPERTIES THAT CAN BE USED TO DIFFERENTIATE PSYCHROTROPHIC BACILLI FROM RAW AND HEAT-TREATED SAMPLES

Microbiological Characteristics	Biochemical Characteristics
Growth on nutrient agar	Starch hydrolysis
Growth on nutrient broth	Gelatin hydrolysis
Growth on 0-5% NaCl broth	Casein hydrolysis
Growth on glucose-nutrient agar	Indole production
Methylene blue reduction	Nitrate reduction
Growth on proteose-peptone agar	Acetylmethylcarbinol production
Utilization of xylose, mannitol, sucrose, lactose, glucose and arabinose	Gram reaction
	Citrate utilization
	Urease activity

(from Grosskopf and Harper, 1974)

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#### Available Facilities and Equipment:

The Technology Unit of the Dairy Foods Division of the Hannah Research Unit, Ayr, Scotland is divided into the Departments of Physical Chemistry and Milk Utilization. The Dairy Foods Division has just completed conversion of a building (formerly used for climatic physiology) as a technical center. The facility is able to duplicate most dairy processes. The equipment and facilities are complemented by the nearby Dairy Technology Department of the West of Scotland Agricultural College. Appropriate laboratories for conducting microbiological and biochemical analyses and protease assays are available.

#### Cooperation

This is a cooperative research project that will be conducted in conjunction with the current research interest and assistance of Dr. M.W. Griffiths, Scientist at the Department of Milk Utilization, Hannah Research Institute, Ayr, Scotland. The principal investigator, F.W. Bodyfelt will conduct much of the research while on sabbatic leave at the Hannah Research Institute in Scotland, November 15, 1987 through May 15, 1988.

Financial Support 1987-88

<u>Cost Item</u>	<u>Request Budget</u>	<u>Approved Budget</u>
Oregon State University		
Graduate Research Assistant - 1.0 FTE	\$7,200	
OPE @ 5%	360	
Tuition remission	5,090	
Personnel subtotal	12,450	
Consumable supplies	3,500	
Travel costs	1,000	
Printing and mailing	150	
Statistical services	150	
Other expenses	<u>1,035*</u>	
Total Operating Budget	\$18,285	\$18,285

- \* Required Laboratory Fee for Visiting Professor (6 months) at the Hannah Research Institute, Ayr, Scotland.

**Project Title:** Rapid Assay for Heat Resistant Microbial Proteinases in Raw Milk by a Simple Casein Denaturation Method.

**Personnel:** F. W. Bodyfelt, Professor, Dept. of Food Science and Technology,  
Oregon State University

Sergio Feijoo, Graduate Student, Department of Food Science and Technology,  
Oregon State University

M. W. Griffiths, Microbiologist in Charge, Department of Food Science,  
Hannah Research Institute, Ayr, Scotland.

**Funding Sources:** Western Dairy Foods Research Center  
Oregon Agricultural Experiment Station

### Objectives:

1. Develop a diffusion casein-agar test capable of quantitating the proteolytic activity exhibited by heat resistant sporeforming bacteria (*Bacillus* sp.) in either raw or pasteurized milk samples that have been subjected to a standardized heat treatment.
2. Determine the appropriate casein fraction and the optimal buffering and suspension systems for the substrate matrix for conduct of the proteinase assay.
3. Determine the optimal conditions for: (a) The initial heat treatment of milk samples, (b) preliminary incubation conditions, and (c) other necessary assay parameters.
4. Develop a method for monitoring populations of *Bacillus* sp. in selected raw milk samples by a combination of preliminary incubation and a dye reduction test.

### Results:

Experiments were conducted to reassess the optimum conditions (temperature and time) for the initial heat treatment of raw milk samples. Results indicated that the use of 75°C for 20 minutes produces higher eventual counts of surviving (and outgrowing) microorganisms than does the standard or conventional 80°C for 10 (or 12) minute treatment (Fig. 1). Furthermore, it was demonstrated that different strains of psychrotrophic bacilli exhibit varied degrees of heat resistance or sensitivity and respond accordingly to the employed heat pre-treatments. Several experiments demonstrated that B-casein is the preferred substrate for assessing the proteinase activity of *Bacillus* sp. Some of the optimum test parameters for *Bacillus* sp. proteinase assays were evaluated and possibly determined: e.g. pH optima (7.25); sample load onto the casein-agar matrix, minimum reaction or development time and a comparison of vertical vs. horizontal approaches for the conduct of enzyme dispersion through an agar-casein matrix.

An improved microbial growth media for enhanced sporulation rates for *Bacillus* sp. was formulated. Initial trials indicated the potential for a combined preliminary incubation of heat treated milk samples, and a simultaneous rapid dye reduction (redox) test for a more rapid, simple, reliable and economical method for monitoring heat resistant spore forming psychrotrophs in either raw or pasteurized milk samples.



Surveys conducted of the majority of Grade A milk producers in Oregon (1986-87) indicated that at least 25% of all producers had high counts for heat resistant psychrotrophic sporeformers (e.g. >100 cfu/ml.). More recently (1988), a majority of the pasteurized milk and cream products in another Oregon survey indicated shelf life limitations probably due to *Bacillus* sp. surviving the pasteurization treatment (Table 2).

### Impact of Research:

Three major milk processors in the Pacific Northwest incurred serious and wide-spread consumer complaints related to "sweet curdle" defects in fluid milk and cream products in January-February, 1989. Numerous school milk quality problems stem from "early" curdling and objectionable off-flavors due to proteinase activity of *Bacillus* sp. derived from raw milk supplies. Cottage cheese and conceivable cheddar cheese yields and product quality can be adversely affected when *Bacillus* sp. and their associated proteinases prevail in milk supplies. The current test method for heat resistant sporeforming psychrotrophs in raw milk sources requires at least 10-12 days to complete. A more rapid method for determining objectional levels of *Bacillus* sp. in raw milk supplies would be a most advantageous analytical tool for inclusion in conventional quality bonus or incentive payment programs.

Conservatively, assuming that approximately one fourth of all milk producers incur higher than desirable *Bacillus* sp. counts, there may be no better way to focus on this ubiquitous milk quality problem than to develop a rapid, routine test method for screening milk samples for this troublesome microflora. Such a reliable and economically feasible analytical tool could be an invaluable step forward for enhancing milk quality at the farm level. An effective tool for rapid and accurate detection of *Bacillus* sp. could serve as a keystone test within milk quality incentive programs for the U. S. dairy industry.

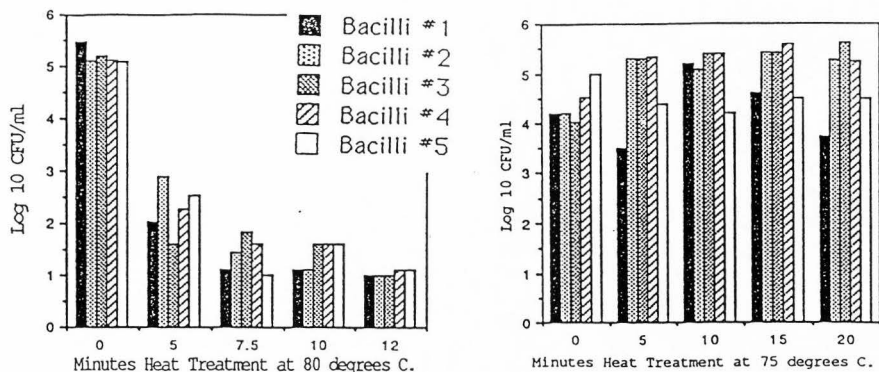


FIG. 1. SURVIVOR SPORE COUNTS FOR 5 STRAINS OF PSYCHROTROPHIC BACILLI

TABLE 1

A SURVEY OF OREGON PASTEURIZED MILK & CREAM PRODUCTS\* FOR THE OCCURRENCE OF SPOREFORMERS AND PRODUCT "SPOILAGE POTENTIAL"

No. of Samples	Spore Cts. >10/ml	Psychro Spore Cts. >10/ml	Flavor "Failure" @ 7°C-days	
			10d	14d
59	33	37	35	40
(100)%	(56)	(63)	(59)	(68)

\*Skim, Low-Fat, Homog. & H&H

**Project Title:** Rapid Assay for Heat-Resistant Microbial Proteases in Raw Milk by a Simple Casein Denaturation Method

**Personnel:** Floyd W. Bodyfelt, Dept. of Food Science & Technology, Oregon State University

Sergio C. Feijoo, Dept. of Food Science & Technology, Oregon State University

Carlos Gonzalez, Dept. of Food Science & Technology, Oregon State University

**Funding:** Western Dairy Foods Research Center and Oregon Dairy Products Commission

**Objectives:**

Heat-resistant sporeforming psychrotrophic bacteria produce proteinases in raw or pasteurized milk that are responsible for causing marked deterioration of milk quality, e.g., bitter taste, unclean off-flavor and/or protein coagulation (sweet curdle). The typical microbiological procedures that are used for isolating, enumerating and confirming the presence of thermophilic psychrotrophs (or their associated proteinases) are laborious, time consuming and do not provide directly information about potential proteolytic activity. Hence, the development and possible adoption of a simple, rapid and sensitive assay for relatively low concentrations of proteinases in raw milk supplies or pasteurized milk products could provide processors with an early awareness or warnings about possible quality problems.

1. Develop a diffusion casein-agar test capable of quantitating the proteolytic activity exhibited by heat-resistant sporeforming bacteria (Bacillus spp.) in either raw or pasteurized milk samples that have been subjected to a standardized heat treatment.
2. Determine the optimal conditions for: (1) the initial heat treatment (standardized) of milk samples, (2) preliminary incubation conditions, and (3) other necessary assay parameters.
3. Determine the most appropriate casein fraction and the optimal buffering and suspension systems for the substrate matrix for conduct of the proteinase assay.
4. Develop an alternative method for determining populations of Bacillus spp. in selected raw milk samples by a combination of preliminary incubation and a dye reduction test.

## Results:

A sensitive diffusion casein-agar method was developed to detect the proteolytic activity exhibited by heat resistant sporeforming bacteria (Bacillus spp.) in raw milk. Initial experiments demonstrated  $\beta$ -casein to be the preferred substrate for assessing proteinase activity of Bacillus spp. Optimum test parameters for proteinase assays were determined; initial heat-treatment of milk samples at 75°C for 20 min.; pH optima (7.25) of the casein-agar matrix; the sample load (15 $\mu$ l); an indication of proteinase activity within 33-36 hrs.; and use of a plate-well method for assessing enzyme dispersion through the agar suspended substrate. To optimize sporulation of potential Bacillus spp. that may have survived the initial heat treatment of raw samples, 1 ml aliquots of each heat-treated milk sample were inoculated into a defined media (nutrient broth with 5 added minerals and 0.05% NDM), followed by 18 hr. incubation at 21°C. Next, 15  $\mu$ l aliquots were added to formed wells (4.0 mm dia.) in Petri dishes (90.0 mm) that contained 15 ml of  $\beta$ -casein-agar matrix and were incubated for 18 hrs at 30°C. The extent of proteinase activity was proportional to the relative zone size of casein precipitation, which closely corresponded with the magnitude of milk sample spore and psychrotrophic spore counts.

Within the course of evaluating the best possible method(s) for assay for bacilli proteinases, experiments and trials were undertaken of a vertical, micro-hematocrit-tube and several dye reduction tests. However, lack of sensitivity, clear-cut endpoints and cumbersome laboratory handling characteristics appeared to preclude either of these methodologies. The plate diffusion assay with appropriate preliminary incubation for proteinase detection appeared to be the most promising technique for assessment of enzyme activity.

## Impact of Research:

Five major milk processors in the Pacific Northwest incurred and reported serious and widespread consumer complaints related to "sweet curdle" defects in fluid milk and cream products in 1989-1991. Also, numerous school milk quality problems stem from "early" curdling and objectionable off-flavors due to proteinase activity of Bacillus spp. derived from raw milk supplies. Cottage cheese, and conceivably cheddar cheese, yields and product quality can be adversely affected when Bacillus spp. and their associated proteinases develop in cheese milk. The current test method for heat-resistant sporeforming psychrotrophs in raw milk sources requires at least 10 or 12 days to complete. A more rapid method for determining objectional levels of Bacillus spp. in raw milk supplies would be a most advantageous analytical tool for inclusion in processor quality incentive (bonus) payment programs. Producer associations and processors across the U.S. have requested information about criteria for thermophilic microflora in raw milk for possible incorporation in milk quality incentive programs.

Conservatively, assuming that approximately one fourth of all milk producers incur higher than desirable Bacillus spp. counts, there may be no better way to focus on this ubiquitous milk quality problem than development and adoption of a rapid, routine test

ethod for screening milk samples for this troublesome microflora. Such a reliable and economically feasible analytical tool could be an invaluable step forward for enhancing milk quality at the farm level. An effective tool for rapid and accurate detection of Bacillus spp. could serve as a keystone test within milk quality incentive programs for the U.S. dairy industry.

#### Publications:

leer, Ralph R. 1988. Identification and characterization of some psychrotrophic heat resistant/sporeforming bacteria in the Grade A raw milk supply of Oregon. M. S. Thesis. Oregon State University, Corvallis, 81 p.

Feijoo, S. C. and F. W. Bodyfelt. 1990. A rapid method for determining proteases of heat-resistant Bacilli in raw milk. Abstract in J. Dairy Sci. 73 (6): Suppl. 1.

leer, R. R., M. J. Woodburn and F. W. Bodyfelt. 1991. Identification and characterization of heat-resistant psychrotrophic bacteria in Oregon Grade A raw milk. Accepted for publication in J. Food Protection, (7/22/91).

leer, R. R., J. Baker, F. W. Bodyfelt, and M. W. Griffiths. 1991. Psychrotrophic Bacillus spp. in fluid milk products: A review. Accepted for publication in J. Food Protection (7/15/91).

Feijoo, S. C. 1991. Rapid assay for Bacillus proteinases in raw milk as detected by a simple casein denaturation method. M. S. Thesis. Oregon State University, Corvallis, 79 p.

Feijoo, S. C., C. Gonzalez, and F. W. Bodyfelt. 1991. A simple casein denaturation method for rapid assay of bacilli proteinases in milk. Manuscript in preparation.

Project Outline

WESTERN STATES DAIRY FOODS RESEARCH CENTER  
(Utah State, Oregon State and Brigham Young Universities)

Project No. \_\_\_\_\_  
Fund: \_\_\_\_\_

Title: Production of Omega-3 Fatty Acids By Genetically  
Altered Fungi and Lactic Acid Bacteria

Personnel: F.W. Bodyfelt, Department of Food Science and Technology,  
Oregon State University  
Sam Beattie, Graduate Research Assistant,  
Department of Food Science and Technology  
W.E. Sandine, Department of Microbiology  
D.P. Selivonchick, Department of Food Science and Technology

Original Starting Date: September 1, 1987

Date of Last Revision

Duration of Current Project September 1, 1987  
Starting Date

August 31, 1991  
Estimated Completion Date

Institutional Units Involved:

Department of Food Science and Technology - Oregon State University  
Department of Microbiology - Oregon State University

Authorized:

_____ Lloyd W. Bodyfelt Project Leader	_____ Date	_____ William E. Sandine Co-Project Leader	_____ Date
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_____ Daniel P. Selivonchick Co-Project Leader	_____ Date
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_____ J.A. Scanlan Department Head Department of Food Science and Technology Oregon State University	_____ Date	_____ George H. Keller Vice-President for Research Oregon State University	_____ Date
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_____ Richard C. Greenwood Director of Business Affairs Oregon State University	_____ Date
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### Justification:

The use of various types of microorganisms to produce human food and related products has a long history. Yeasts, bacteria and molds have all been utilized in designed biomass conversions to produce commercially valuable end products. With the advent of genetic engineering it has become possible to remove DNA from one microorganism and recombine it with the DNA from another organism. This DNA transfer can provide a combination of desirable genetic traits within the "recipient" microorganism. Additionally, recombinant DNA technology allows construction of microorganisms that are able to grow on normally unusable substrates and also to produce substances foreign to the organism. An example of this technology is production of mammalian insulin by an unlikely organism, Escherichia coli.

Under-utilized by-products or waste materials from both food and non-food processing operations are readily available as growth substrates for microorganisms. Good examples of this are the production of single cell protein from sulfite waste liquor (from paper manufacture) and ethanol production from lactose fermentation of cheese whey. Whey is quite high in organic material, which results in high biological oxygen (BOD) and chemical oxygen demand values (COD). These properties and the high volumes generated combine to make whey disposal difficult and relatively expensive (ten kg of cheese milk results in merely one kg of cheddar cheese, but approximately 9 kg of whey). Because of the high BOD and the dilute nature of cheese whey, it must ordinarily be disposed of by means other than through release to sewage systems.

Various methods of isolating, concentrating and drying the proteins of cheese whey have been developed, as well as fermentation of the lactose fraction into ethanol by genetically altered Saccharomyces sp. yeasts (W. B. Gibbons, SDSU ethanol pilot project, personal communication). Hammond and coworkers (Moon et al. 1978; 1978b) isolated yeasts that could utilize cheese whey to produce high levels of lipid. Surprisingly, these oleaginous yeasts could produce up to 57% oil (equivalent dry weight of cells) from the permeate of ultrafiltered whey. The ultrafiltration process on whey primarily removes those proteins above 24 kd, thus leaving lactose, peptides and assorted low molecular weight molecules and ions in the permeate. Lipid produced in this manner was high in oleic (18:1) and palmitic (16:0) acids. Furthermore, this method of processing reduced the COD of the whey by 70-90%.

Lipid production by yeasts cannot be considered an economical means of disposing of cheese whey, given the "fatty acid profile" of this oil product. However, the current interest of nutrition scientists in the health promotion role of long chain polyunsaturated fatty acids (PUFA's; e.g., 5, 8, 11, 14, 17-eicosapentaenoic acid [EPA] and 4, 7, 10, 13, 16, 19-docosahexenoic acid [DHA]) (Kinsella, 1987; Kinsella, 1986; Newsweek, 1987) suggests that commercial production of these omega-3 fatty acids may become economically desirable. Production of omega-3 fatty acids in milk products offers potential opportunity for increased dairy product sales.

Currently, the major sources of omega-3 fatty acids for human consumption are fish and fish oils (Kinsella, 1987). This is the result of food chain interactions that involve plankton and other primary producers that synthesize substantial amounts of omega-3 fatty acids. Additionally, some lower fungi are capable of producing relatively high quantities of EPA.

This research project proposes to use whey permeate as a growth medium for PUFA-producing fungi that have been genetically altered by cloning lactose metabolizing genes. This development would enable biomass production on whey. Eventually, efforts will be made to clone the PUFA-producing genes into lactic acid bacteria of the Streptococcus, Lactobacillus, Leuconostoc and Pediococcus genera. The use of such altered bacteria in fermented or sweet (unfermented) milk products would provide a vehicle for consuming dairy products enriched for these healthful omega-3 fatty acids. Consumers in the United States are constantly being made aware of the cholesterol-reducing benefits of certain foods (e.g. fish) that contain omega 3-fatty acids. Appropriate promotion and advertising of any milk products that contained lactic acid bacteria which synthesize these acids could conceivably lead to increased consumption of milk and milk products. Combining such lactic organisms with yeasts that had been modified to produce omega-3 fatty acids would also permit the production of kefir or other cultured dairy foods with substantially enhanced health promoting characteristics.

#### Previous Work and Present Outlook

Saprolegnia parasitica is an aquatic fungus that is commonly associated with diseased fish and fish eggs (Powell et al., 1972; Alexopoulos and Mims, 1979). This fungus belongs to the class Oomycetes and is characterized by motile zoospores (Alexopoulos and Mims, 1979). Powell et al. (1972) in a comprehensive study of the nutritional requirements of S. parasitica developed a basal medium that contained sodium glutamate as the sole carbon /nitrogen source. Additional carbon sources were added to this basal medium to determine if growth of S. parasitica was improved. The organism was unable to utilize lactose as a carbon source. When galactose was incorporated into the basal medium, growth was slightly improved; glucose was the preferred substrate for optimum growth. Higher cell yields were realized when casein hydrolysate was used as the nitrogen source.

Interestingly, Gellerman and Schlenk (1979) discovered that S. parasitica produced relatively high amounts of EPA when grown on the glucose based medium of Powell et al. (1972). The organism was found to contain 13% lipid (on a dry weight basis) with EPA making up 20% of the fatty acid profile. The glucose medium used in this study provided sufficient nitrogen. Work in Ratledge's laboratory (Boulton and Ratledge, 1983; Evans and Ratledge, 1984, Gill et al., 1977) has demonstrated that fungi grown under conditions of limited nitrogen, shift metabolism from protein and nucleotide biosynthesis to lipid accumulation. These results occurred when the carbon source was not limiting.



We hypothesize that *S. parasitica* lacks genes to produce the enzymes for metabolism of lactose and galactose. Since one goal of this research is to develop an organism that can utilize whey permeate, it will be necessary to introduce the proper lactose metabolizing genes into *S. parasitica*. Introduction of foreign DNA into fungi with successful expression of the genes has been accomplished (Spencer and Spencer, 1983; Turner et al., 1985; Ullrich et al., 1985). In this regard, the genes for lactose metabolism are perhaps the most studied and characterized in both prokaryotic and eukaryotic systems.

A major difficulty in the genetic alteration of fungi with exogenous DNA has been the lack of a shuttle vector that is able to replicate in *E. coli* and fungi. Lambowitz and coworkers (Collins et al. 1981; Stohl and Lambowitz, 1983; Stohl et al., 1982) used a mitochondrial plasmid of *Neurospora crassa* to produce a recombinant plasmid that was able to replicate autonomously in *E. coli* and *Neurospora*. van Gorcom et al. (1985) successfully transformed *Aspergillus nidulans* by means of a plasmid that contained *E. coli lacZ* coding for  $\beta$ -galactosidase. The *E. coli* gene was cloned into the *trpC* gene of *A. nidulans* and used the *trpC* translation and transcription start sequences of the fungus. These studies indicate that transformation of fungi is accomplished more efficiently by use of plasmids constructed from host plasmid DNA that contains a promoter from the host.

The genetics of *Saprolegnia* sp. have not been examined extensively and at this point it is not known if the organism contains extrachromosomal DNA other than that found in the mitochondria. Based on research with higher fungi (*Neurospora*, *Penicillium* and *Aspergillus*), extrachromosomal plasmid DNA is likely to be found in *S. parasitica*. Techniques used in protoplast fusion of plants and yeasts should find application in this research. In the likely event that the transformation of *S. parasitica* to a lactose metabolizing organism is successful, a new and economically advantageous means of using whey permeate could be available. The fact that *S. parasitica* contains a relatively simple genome should facilitate the identification of genes responsible for omega-3 fatty acids production.

### Objectives

1. Examine *Saprolegnia parasitica* for extrachromosomal DNA/plasmids with the goal of using an indigenous plasmid for cloning genes facilitating metabolism of lactose.
2. Once genetically altered, determine growth and lipid accumulation (fatty acid profile) of *S. parasitica* in a chemostat using lactose as a carbon source.
3. Develop a whey permeate based medium that will provide optimum growth and lipid accumulation by *S. parasitica*.
4. Determine the scale-up economics with an emphasis on optimum lipid extraction from large scale chemostat production of *S. parasitica*.
5. Clone genes for omega-3 fatty acid production into lactic acid bacteria.

## Procedures

A goal of this research is to produce a lactose utilizing organism that can accumulate PUFA's when grown on whey permeate. The organism that we propose to genetically alter, *S. parasitica*, does not have the ability to utilize lactose; this feature provides a marker for determining if genetic manipulations are successful.

The genetics of *S. parasitica* have not been examined to a great extent, therefore it will be necessary to examine the organism for indigenous plasmid DNA. The successful plasmid separation techniques used by Lambowitz and coworkers (Collins et al. 1981; Stohl and Lambowitz, 1983; Stohl et al., 1982) in isolating plasmid DNA from mitochondria of *Neurospora* will be used initially for *S. parasitica*. This technique involves centrifugation of mitochondrial lysates in cesium chloride-ethidium bromide gradients. Additionally, we will look for plasmids of non-mitochondrial origin in whole protoplast lysates. The size of plasmids will be determined by restriction analysis using standard methods (Maniatis et al., 1982).

Ideally, a shuttle vector will be constructed from a plasmid found in the fungus and a plasmid capable of replicating in *E. coli*. Stohl and Lambowitz (1983) describe construction of a shuttle vector plasmid using a *Neurospora* plasmid cloned into pBR325, an *E. coli* plasmid. This chimeric plasmid was able to replicate in either organism.

In our research, an *E. coli* plasmid that contains *lacZ* will be used as a cloning vector for the fungal plasmid. This should provide a plasmid that will replicate in either organism. van Gorcom et al. (1985) demonstrated that plasmids constructed in this manner will express the desired gene product using the fungal translation/transcription start point. The use of the chromogenic substrate XGal (5-bromo-4-chloro-3-indole- $\beta$ -D-galactoside) in the fungal medium (Casadaban et al., 1983) will facilitate selection of transformed colonies (blue) of *S. parasitica* that are able to metabolize lactose. Antibiotic resistance will serve as the marker for transformed *E. coli*.

Once successfully transformed, the physiological requirements of the fungus will be examined with respect to growth in a chemostat. At this stage a whey permeate based medium will be developed. Ideally, this medium should provide a ready carbon source (lactose) and require limited supplementation. With respect to PUFA accumulation, optimization of pH, temperature, flow rates, nitrogen source and concentration, and other parameters as discussed by Moon et al. (1978) will be followed by GC analysis of the lipids of *S. parasitica*. Fatty acid methyl esters (FAME's) will be made and separated by means of capillary column gas chromatography procedures currently being followed in Selivonchick's laboratory (personal communication). Various extraction schemes will be examined in light of potential scale-up restrictions, i.e. solvent costs, removal of lipid from cell, oxidation problems, etc.

The cloning of the omega-3 fatty acid genes into lactic acid bacteria would proceed on the assumption that these genes are located on a chromosome of S. parasitica. It would be necessary to develop an assay to recognize clones (colonies) of organisms that contain these genes. Since expression of the genes likely would result in production of large amounts of the omega-3 fatty acids, clones may be recognized by a different colonial morphology. Replicated plates flooded with copper sulfate may also be used to identify fatty acid producing clones, as done to distinguish lipase producing Pseudomonas organisms on milkfat agar. In order to develop a suitable assay, mutants of S. parasitica could be induced that lack the ability to synthesize omega-3 fatty acids and then compared to colonies of mutants and wild type cells by the above suggested procedures.

Once an appropriate assay is developed, the genes responsible for omega-3 fatty acid synthesis could be cloned into lactic acid bacteria by using state-of-the-art recombinant DNA techniques. For example, commercially available lambda ( $\lambda$ ) bacteriophage with lacZ inserts could be restricted and ligated with similarly restricted S. parasitica chromosome DNA fragments. Infection of E. coli with resulting  $\lambda$ -phage would yield blue and colorless plaques on X-gal medium. The latter response would be an indication of insertional inactivation. Phages from such colorless (clear) plaques could be isolated and propagated and DNA produced therefrom be used for transfection of E. coli to amplify omega-3 fatty acid coding  $\lambda$ -fragments. This DNA, which would contain the desired genes, might then be inserted into a promiscuous vector, such as pBR322, for cloning into E. coli cells, and subsequently into lactic acid bacteria by plasmid DNA transformation.

Since eukaryotic cells (yeast) have introns (non-coding DNA sequences), it is possible that the S. parasitica genes will not be expressed in bacterial systems. Therefore, another cloning strategy that could be used is isolation of the mRNA of the omega-3 fatty acid genes and then use reverse transcriptase to produce the appropriate cDNA, which could then be cloned into E. coli or lactic acid bacteria.

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**Project Title:** Production of Omega-3 Fatty Acids by Genetically Altered Fungi and Lactic Acid Bacteria.

**Personnel:** F.W. Bodyfelt, Professor, Dept. of Food Science and Technology, Oregon State University

D.P. Selivonchick, Professor, Dept. of Food Science and Technology, Oregon State University

S.E. Beattie, Graduate Research Assistant, Dept. of Food Science and Technology

W.E. Sandine, Professor, Microbiology, Oregon State University.

**Funding Sources:** Western Dairy Foods Research Center  
Oregon Agricultural Experiment Station

**Research Objectives:**

1. Examine *Saprolegnia parasitica* for plasmids with the goal of using an indigenous plasmid for cloning purposes.
2. Once genetically altered, determine growth and lipid accumulation of *S. parasitica* in a chemostat using lactose as a carbon source.
3. Develop a whey permeate based medium that will provide optimum growth and lipid accumulation.
4. Determine scale up economics with an emphasis on optimizing lipid extraction from large scale chemostat production of *S. parasitica*.
5. Begin work towards construction of strains of lactic acid bacteria and yeasts that are able to produce omega-3 fatty acids.

**Results:**

The objectives of this work are to examine the potential use of cheese whey permeate as a growth substrate for *Saprolegnia parasitica*. *S. parasitica*, when grown on a monosaccharide substrate, produces omega-3 fatty acids. Since the organism is unable to utilize lactose, we are concentrating on cloning lactose metabolizing genes into *S. parasitica*.

Initial studies have examined lipid profiles of *S. parasitica* grown in media limiting the carbon and nitrogen sources. The defined medium that showed optimum omega-3 fatty acid (eicosapentenoic acid {20:5  $\Delta$ 5,8,11,14,17}, EPA) production contained adequate carbon and nitrogen as glutamic acid and no glucose. When grown on this medium at 20 °C, the fatty acid profile of *S. parasitica* was 24.69% EPA. When a casein hydrosylate was used as the sole nitrogen source, EPA production averaged 18.4% of the total fatty acid profile. Limiting the nitrogen source adversely affected the total growth and EPA production of *S. parasitica*, but increased total lipid production to 4.0% (wet weight basis). This data provides a baseline for further studies using genetically altered *S. parasitica*.

Since *S. parasitica* does not contain enzymes necessary for lactose metabolism, it is necessary to genetically transform the organism. The first step to this is formation of stable protoplasts that are able to regenerate cell walls and form normal hyphae. Stable protoplasts have been generated by use of a combination of enzymes and osmotically stabilized with 0.5 M KCl or 1 M sorbitol. With this method, approximately 20% of the protoplasts regenerate. More recently 0.6 M sorbitol was found to stabilize protoplasts and allow higher regeneration percentages.

Initial plans were to use a mitochondrial plasmid from *S. parasitica* as with a portion of a vector plasmid that would contain the genes for lactose metabolism. Based on electrophoretic analysis, no mitochondrial plasmids have been found. Therefore, a 'shotgun' approach has been employed using chromosomal DNA and various plasmids.

Within the last month we have successfully transformed *S. parasitica* to lactose utilization. Plasmid pKRIB-Lac4-1 (kindly supplied by R. Dickson) was used to transform *S. parasitica* by a polyethylene glycol procedure. This plasmid contains the B-galactosidase and lactose permease genes as well as an ARS section of the yeast *Kluyveromyces fragilis*. Transformed protoplasts were identified by their ability to grow and produce blue colonies on a lactose based medium that contained the chromogenic indicator X-gal. Relatively high concentrations of plasmid DNA are required to achieve transformation and only 10-15 transformants/10<sup>6</sup> protoplasts have been obtained. Colony growth is very poor on the regeneration medium currently in use. Work to improve transformant growth and identification of the mode of integration of pKRIBLac4-1 into *S. parasitica* are in process. Future experiments will focus on lactose uptake and whether the permease is needed. Development of a whey permeate medium is planned in an effort to optimize lipid profiles.

### Economic Impact:

While there is some discussion of the health promoting benefits of PUFAs (especially omega-3), it is generally accepted that a diet with a high unsaturated to saturated fat ratio is beneficial. Some health experts encourage the addition of omega-3 fatty acids supplements, in the form of fish oil gelcaps (MaxEPA, Promega, etc), to normal diets. Additionally, it has been discovered that some animals when fed omega-3 fatty acids are able to incorporate them into tissue fats. Hens fed fish meal tend to lay eggs high in omega-3 fatty acids. With markets for omega-3 fatty acids increasing, a new source of these fats could have an economic impact within the scope of the dairy industry. The most troublesome byproduct of cheese manufacture is whey permeate. Because of residual lactose, this byproduct is high in biological oxygen demand (BOD) and therefore expensive to dispose of through common means. A process that could reduce the BOD and simultaneously produce a marketable product would be beneficial to cheese manufacturers. This research is designed to fulfill that need by producing microbial oil that is rich in omega-3 fatty acids making it comparable to salmon or other fish oils.

**Project Title:** Production of Omega-3 Fatty Acids by Genetically Altered Fungi and Lactic Acid Bacteria

**Personnel:** Floyd W. Bodyfelt, Principal Investigator, Professor, Dept. of Food Science and Technology, Oregon State University

William E. Sandine, Co-Investigator, Professor, Dept. of Microbiology, Oregon State University

Daniel P. Selivonchick, Co-Investigator, Professor, Dept. of Food Science and Technology, Oregon State University

Sam E. Beattie, Graduate Research Assistant, Dept. of Food Science and Technology, Oregon State University

**Funding Sources:** Western Dairy Foods Research Center

#### Objectives:

1. Examine Saprolegnia parasitica for extrachromosomal DNA/plasmids with the goal of using an indigenous plasmid for cloning genes facilitating metabolism of lactose.
2. Once genetically altered, determine growth and lipid accumulation (fatty acid profile) of S. parasitica using lactose as a carbon source.
3. Develop a whey permeate based medium that will provide optimum growth and lipid accumulation by S. parasitica.
4. Determine the scale-up economics with an emphasis on optimum lipid extraction from large scale chemostat production into lactic acid bacteria.

#### Results:

This research examines an approach to producing PUFAs from fungi genetically altered to lactose utilization. Saprolegnia parasitica, a filamentous fungus, was examined for eicosapentaenoic acid (20:5 omega-3, EPA) production when grown on six media with varied nitrogen and carbon sources. Optimum EPA production reached 24% of total fatty acids. In an effort to produce EPA from lactose, transformation experiments using the plasmid pKRI BLAC4-1 were undertaken. Transformation of protoplasts was by PEG/CaCl<sub>2</sub>

with 1 to 6 protoplasts/mg DNA transformed. Several stable transformants were obtained. As indicated by Southern hybridization, the plasmid was incorporated genomically. SP829 produced 4-5 units  $\beta$ -galactosidase/mg protein.  $^{14}\text{C}$  lactose was used to determine lactose uptake by SP829 mycelia and protoplasts. Radiolabel was not detected in protoplasts or mycelia. This indicates that while  $\beta$ -galactosidase is produced the fungus lacks a functional lactose permease.

### Impact of Research:

There is good evidence that the omega-3 fatty acids (PUFAs) found in certain fish oils have health promoting properties. Production of omega-3 fatty acids by a microorganism able to grow on cheese whey permeate would be economically beneficial to the dairy industry for several reasons. Disposal of whey permeate by cheese processors can be costly. A 70-90% reduction in the BOD of whey permeate might be expected by an organism that could produce oil from the organic material (lactose, peptides) in whey permeate. This could markedly reduce sewage disposal costs for cheese processors. Another economic benefit from a "dairy system" production of omega-3 fatty acids is provision of a valuable byproduct from whey permeate. Current nutrition research and related health claims indicate that increased consumption of omega-3 fatty acids could be quite beneficial to humans. When fed to animals, omega-3 fatty acids are deposited into strategic animal tissues, thereby improving the fatty acid profile. This and subsequent research on the genetics of omega-3 fatty acid producing microorganisms may facilitate cloning of these genes into dairy starter cultures. This could improve the fatty acid profile of fermented dairy products. Such a technological development may enhance the sales of fluid milk and milk products.

### Publications and Abstracts:

Beattie, S.E., W.E. Sandine, D.P. Selivonchick and F.W. Bodyfelt. 1990. Transformation of an omega-3 fatty acid producing fungus with LAC4 ( $\beta$ -galactosidase) of Kluyveromyces lactis. Abstract in J. Dairy Science. 73(6).Suppl.1.

### Patents:

N/A



**Project Title:** Production of Omega-3 Fatty Acids by Genetically Altered Fungi and Lactic Acid Bacteria

**Personnel:** Floyd W. Bodyfelt, Dept. of Food Science & Technology, Oregon State University

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Daniel P. Selivonchick, Dept. of Food Science & Technology, Oregon State University

William E. Sandine, Dept. of Microbiology, Oregon State University

**Funding:** Western Dairy Foods Research Center

**Objectives:**

Examine S. parasitica for extra chromosomal DNA/plasmids with the goal of using an indigenous plasmid for cloning genes facilitating metabolism of lactose.

Since S. parasitica does not contain the enzymes necessary for lactose metabolism, it was necessary to genetically transform the organism. The first step to this was formation of stable protoplasts which were generated by use of a combination of enzymes and osmotic stabilization with 0.5M KCl (or 1M sorbitol). With this method, approximately 20% of the protoplasts regenerated. Subsequently, 0.6M sorbitol was found to stabilize protoplasts and allow higher regeneration percentages.

Finally, we successfully transformed S. parasitica to utilize lactose. Plasmid pKR1B-Lac4-1 (supplied by R. Dickson, U. of Kentucky) was used to transform S. parasitica by a polyethylene glycol procedure. This plasmid contains the  $\beta$ -galactosidase and lactose permease genes as well as an ARS section of the yeast Kluveromyces fragilis. The transformed protoplasts were identified by their ability to grow and produce blue colonies on a lactose-based medium that contained the chromogenic indicator X-gal. Relatively high concentration of plasmid DNA were found to be required to achieve transformation and only 10-15 transformants for every  $1 \times 10^6$  protoplasts were obtained. Colony growth was quite poor on the regeneration medium.

The majority of the research was involved in the genetic alteration of S. parasitica. A transformation procedure was developed that allowed selection of transformants of S. parasitica based on the production of  $\beta$ -galactosidase. Because the plasmid DNA did not incorporate into chromosomal DNA, transformants were unstable. While transformants were shown to be unstable, the

transformation procedure should work for plasmids that incorporate into chromosomal DNA. A plasmid based on the trpC promoter of Aspergillus nidulans may lead to transformants.

2. Once genetically altered, determine growth and lipid accumulation (fatty acid profile of S. parasitica in a chemostat using lactose as a carbon source.)

Initial studies examined lipid profiles of S. parasitica grown in media of limited carbon and nitrogen sources. The defined medium that showed optimum omega-3 fatty acid (eicosapentenoic acid {20:5Δ5,8,11,14,17}, EPA) production contained adequate carbon and nitrogen as glutamic acid and no glucose. When grown on this medium at 20°C, the fatty acid profile of S. parasitica was 24.6% EPA. When a casein hydrosylate was used as the sole nitrogen source, EPA production averaged 18.4% of the total fatty acid profile. Limiting the nitrogen source adversely affected the total growth and EPA production of S. parasitica, but increased total lipid production to 4.0% (wet weight basis). This data provided a baseline for further studies using genetically altered S. parasitica.

3. Develop a whey permeate based medium that will provide optimum growth and lipid accumulation by S. parasitica.

A most troublesome byproduct of cheese manufacture is whey permeate. Because of residual lactose, this byproduct is high in biological oxygen demand (BOD) and therefore expensive to dispose of through common means. A process that could reduce the BOD and simultaneously produce a marketable value-added product would be beneficial to cheese manufacturers. An intent of this research was to fulfill that need by possibly producing microbial oil that would be rich in omega-3 fatty acids; thus making it comparable to salmon or other fish oils.

One of the major objectives of this work was to examine the potential use of cheese whey permeate as a growth substrate for Saprolegnia parasitica. S. parasitica, when grown on a monosaccharide substrate, produces omega-3 fatty acids. Since the organism is unable to utilize lactose, much effort was expended on trying to clone lactose metabolizing genes into S. parasitica.

Since the transformants of S. parasitica exhibited instability (because the plasmid DNA did not incorporate into chromosomal DNA), the achievement of this objective was not consummated.

4. Determine the scale-up economics with an emphasis on optimum lipid extraction from large scale chemostat production into lactic acid bacteria.

The constraints and limits of achieving the first two objectives of this study made this objective superfluous. Also, recent Food and Drug Administration restrictions on making any health claims for omega-3 fatty acid profiles in food products served to lessen the nutritional impact of this approach.

## Results:

Lipid production by microorganisms cannot be considered an economical means of disposing of cheese whey permeate when the lipid composition is similar to that of seeds. However, the current interest of nutritionists in the health promotion role of long chain polyunsaturated fatty acids (PUFAs), in particular the omega-3 fatty acids (e.g. 8,11,14,17-eicosapentaenoic acid (EPA)), suggests that commercial production of omega-3 fatty acids may be economically feasible. Relatively few microorganisms are able to produce omega-3 fatty acids; exceptions are phytoplankton and several filamentous fungi. The lower aquatic fungus, Saprolegnia parasitica, has been found to have a fatty acid profile that contains 20% EPA. Unfortunately, S. parasitica does not utilize lactose.

This research examined the ability of S. parasitica to produce omega-3 fatty acids with an emphasis on optimizing the yields of this PUFA by medium manipulation. We successfully transformed the organisms with the LAC5 ( $\beta$ -galactosidase) gene of Luyveromyces lactis by use of the plasmid pKR1B-LAC4-1. Unfortunately, the resultant transformants were unstable and lost the capability to produce  $\beta$ -galactosidase after several transfers over a period of several months.

## Impact of Research:

The primary objectives of this research were to develop an organism that would produce omega-3 fatty acids when grown on a medium that contained lactose (e.g. whey permeate). Saprolegnia parasitica was shown to produce an omega-3 fatty acid when grown on a medium that contained glucose. The influence of medium composition with respect to carbon and nitrogen source showed that optimum lipid and omega-3 fatty acid production occurred in a medium that contained glutamate as carbon and nitrogen sources. Omega-3 fatty acid production was also favored at 20°C regardless of the medium composition. Overall unsaturation also increased with decreased temperature. The major fatty acids effected by temperature were oleic, palmitic, and eicosapentaenoic acids. The data indicated that deactivation because of temperature may occur at the  $\Delta^{12}$ -desaturase leading to omega-3 fatty acids.

The economic considerations of this project are substantial. Lactose and cheese whey permeate are an economic burden to cheese producers and a potential environmental burden for the community. Production of microbial lipids from whey permeate has been shown to be feasible, provided the lipid can compete with traditional sources. In 1986, capsules of omega-3 fatty acid containing oil were selling for \$0.33 to \$0.66/gram (\$327-665/kg) fatty acid (D.H.S. Greene, personal communication). At that time, these figures made production of oil that contained omega-3 fatty acid economically desirable and feasible. More recently, the effectiveness of omega-3 fatty acids in control of various heart diseases has come under close scrutiny. In the past year the Food and Drug Administration has stopped manufacturers of fish oils from making health claims. Regardless of this action, scientific and epidemiological evidence shows that intake of omega-3 fatty acids can benefit cardiovascular health.

## Publications:

- Beattie, S. E., W. E. Sandine, D. P. Selivonchick and F. W. Bodyfelt. 1990. Transformation of an omega-3 fatty acid producing fungus with LAC4 ( $\beta$ -galactosidase of Kluyveromyces lactis). Abstract in J. Dairy Sci. 73(Suppl. 1):71.
- Beattie, S. E. 1990. DNA transformation of Saprolegnia parasitica, an omega-3 fatty acid producing fungus, with the  $\beta$ -galactosidase gene of Kluyveromyces lactis. PhD Dissertation, Oregon University, Corvallis, 89 p.
- Beattie, S. E., D. P. Selivonchick, W. E. Sandine and F. W. Bodyfelt. 1991. DNA transformation of an omega-3 fatty acid producing fungi (Saprolegnia parasitica) with the  $\beta$ -galactosidase gene of Kluyveromyces lactis. Submitted to J. Am. Oil Chem. Soc.

received

MAY 18 1987

Project Outline

WESTERN STATES DAIRY FOODS RESEARCH CENTER

Project No. 272

Fund:

Title: Cloning the Nisin and other Genes of Lactic Streptococci into Leuconostoc  
Species and Amplification of Nisin Production.

Personnel: W. E. Sandine, Department of Microbiology, Oregon State University  
J. K. Kondo, Department of Nutrition and Food Science, Utah State University

Original Starting Date:

July 1, 1987

Date of Last Revision:

Duration of Current Project:

July 1, 1987

Starting Date

June 30, 1992

Estimated Completion Date

Institutional Units Involved:

Department of Microbiology - Oregon State University  
Department of Nutrition and Food Science - Utah State University

Authorized:

W. E. Sandine  
Project Leader

5/1/87  
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Date

## Justification

Leuconostoc organisms are important members of mixed strain lactic streptococcal starter cultures used in the production of fermented milks and cheeses. They ferment milk citrate and produce carbon dioxide and diacetyl. The diacetyl produced is an important flavor component of cultured buttermilk and sour cream and also of unripened soft cheeses such as cream cheese and cottage cheese. The carbon dioxide produced is important in eye formation in Gouda type cheeses and it also contributes to the effervescent properties of cultured buttermilk. While considerable research on the genetics of lactic streptococci has been carried out, very little is known about Leuconostoc. They deserve more research attention because the genetic manipulation of lactic acid bacteria will have a major impact on food fermentations in terms of strain selection, performance, accelerated cheese ripening, food safety, and new product formulations.

The strategy to be used in this research will be to genetically engineer strains of Leuconostoc which will have more widespread use in milk fermentations. The availability of such strains to be used in starter cultures will make possible the manufacture of specialty cheeses to expand the market of dairy products. Large quantities of these types of cheeses are now imported from Europe. Techniques to introduce genes by conjugation into Leuconostoc spp. have just been developed in Dr. Sandine's laboratory and as a result, accomplishing the objectives outlined below has just now become possible. Fulfillment of the objectives also will allow enhanced use of Leuconostoc in dairy starter cultures and their genetically improved fermentation characteristics will make it possible to produce new and

improved fermented milk products.

### **Previous work and present outlook.**

Leuconostoc is a genus of fastidious gram positive bacteria of the family Streptococcaceae; they are found in fermenting vegetables, dairy products, wine and in sugar refineries (Garvie, E.I., 1974. Genus II Leuconostoc van Tieghen 1878, p. 510-513. In R. E. Buchanan and N. E. Gibbons (eds.) Bergey's Manual of Determinative Bacteriology, 8th Edition, Williams and Wilkins Co., Baltimore). Only a few reports occur in the literature on the genetics of these bacteria. O'Sullivan and Daly (Irish J. Food Sci. Technol. 6:206, 1982) were first to note the presence of plasmids in Leuconostoc. Orberg and Sandine (Appl. Environ. Microbiol. 48:1129, 1984) confirmed the common occurrence of plasmid DNA in Leuconostoc. These authors also noted that members of this genus are quite resistant to the antibiotic vancomycin. The minimum inhibitory concentration of vancomycin for strains of Leuconostoc is greater than 500 micrograms per ml and for some strains, greater than 2,000 micrograms per ml. This discovery made it possible for these workers to develop a conjugation system which has allowed the transfer of certain plasmid-coded genes to recipient Leuconostoc organisms from lactic streptococci. The gene for nisin production has been transferred (Tsai and Sandine, Appl. Environ. Microbiol. 53:352, 1987) as well as the genes for lactose fermentation (Tsai and Sandine, J. Ind. Microbiol. In Press, 1987). Strains of Leuconostoc containing the lactose-fermenting genes from lactic streptococci however are still unable to coagulate milk. This is because they are not sufficiently proteolytic to obtain needed nitrogen from milk protein. Therefore, it will be important to insert protease genes into

Leuconostoc to make them fast acid producers. In this regard, Kok et al. (Appl. Environ. Microbiol. 50:94, 1985) have cloned a HindIII fragment of a Streptococcus cremoris Wg2 plasmid coding for protease production (pWV05) into Bacillus subtilis and S. lactis. Transformed protoplasts of slow S. lactis strains became fast acid producers in milk upon acquisition of the cloned protease genes. Furthermore, the HindIII fragment was a useful probe for the detection of several other lactic streptococcal protease plasmids. This probe will be useful in detecting protease genes in both plasmid and chromosomal DNA. To our knowledge, there are no other reports in the literature concerning the genetics of Leuconostoc.

The research proposal is based on the availability of working conjugation systems between lactic streptococci and Leuconostoc and the likely successful application of gene transfer systems and cloning techniques to construct strains of Leuconostoc with improved milk fermentation characteristics. It has also been discovered in Dr. Sandine's lab that Listeria monocytogenes is inhibited by nisin. With the ability to transfer and localize the nisin genes, the application of these findings to inhibit Listeria in dairy products will also be pursued. Studies are also proposed to examine production of nisin at the molecular level.

### Objectives:

1. To produce and characterize lactose positive Leuconostoc transconjugants obtained by conjugal matings between S. lactis and Leuconostoc spp.
2. To develop transformation and gene cloning systems in Leuconostoc.
3. To introduce into Leuconostoc, plasmid-coded protease genes from lactic streptococci (e.g. S. cremoris Wg2).



4. To use the genetically constructed fast acid-producing Leuconostoc (lactose positive and protease positive) to produce different fermented dairy products such as cultured buttermilk, cottage and Gouda cheese.
5. To conjugally transfer plasmid-coded nisin genes from S. lactis 7962 to Leuconostoc and to study this plasmid at the molecular level.
6. To amplify nisin production by gene cloning techniques.
7. To use nisin producing Leuconostoc in Swiss cheese manufacture to inhibit gas producing anaerobic spore formers such as Clostridium tyrobutyricum.
8. To study the inhibition of L. monocytogenes by nisin and to use genetic engineering techniques to maximize its useful application.

#### Procedures:

**Conjugation and plasmid DNA isolation.** Experience gained to date in our laboratory studying the genetics of Leuconostoc will be of great benefit to this project. As cited above, we have published methods to isolate plasmid DNA from both Leuconostoc and lactic streptococci, and have developed conjugation procedures to transfer the lactose-fermenting genes and the nisin producing genes from S. lactis to Leuconostoc. These procedures, modified as necessary, will continue to be used for conjugation experiments. Specifically, the nisin gene from S. lactis 7962, the lactose utilization genes from S. lactis C2, and the protease genes from S. cremoris Wg2 (or other appropriate donor strains) will be conjugally transferred to Leuconostoc. Confirmation of Leuconostoc transconjugants will be based on the vancomycin and azide resistance of these organisms and the susceptibility of donor, recipient and transconjugants to specific lactic streptococcal phages. Leuconostoc strains to be used as recipients in conjugal matings will be characterized for levels of B-galactosidase and phospho-B-

galactosidase enzymes prior to genetic studies so that acquisition of the lactose utilization genes by recipient cells can be confirmed. Standard plasmid curing techniques such as treatment of cells with high temperature, acridine dyes, and novobiocin will be used, along with plasmid analysis, to determine transfer of specific plasmids. Once plasmids have been identified, the specific genes involved will be examined at the molecular level.

**Cloning and molecular characterization of genetic determinants.** Once specific plasmids containing genes of interest have been identified by conjugation experiments, they will be characterized at the molecular level. First, restriction endonuclease maps of plasmids will be obtained. The location of the genes of interest will be determined using standard techniques such as transposon mutagenesis with Tn919, in vitro deletion analysis using nuclease Bal31 or exonuclease III, and gene cloning. Once the locations of the genes of interest have been determined, they will be subcloned into appropriate hosts. The genes will then be transferred into Leuconostoc by appropriate gene transfer systems to examine gene expression and amplification. In order to clone genes directly into Leuconostoc, protoplast transformation systems will also be developed.

**Gene cloning strategies.** All cloning experiments will employ standard methods for restriction enzyme digestions and either cohesive or blunt-end ligations. Cloning strategies will attempt to: (i) clone fragments into streptococcal vectors (pGB301) and transform S. lactis or Leuconostoc directly (Kondo and McKay, Appl. Environ. Microbiol. 48:252); (ii) clone desired fragments in E. coli (Dao and Ferretti, Appl. Environ. Microbiol. 49:115, 1985) or Bacillus subtilis (Kok et al., Appl. Environ. Microbiol., 48:726, 1984) recipients using streptococcal shuttle vectors (pSA3, pGK12, or

pGK13) followed by transformation of appropriate cloned DNA into expression hosts (S. lactis or Leuconostoc). Expression of genes will be evaluated in all recipient strains. S. lactis and/or Leuconostoc strains possessing new capabilities will then be tested in pilot plant studies. Appropriate cloning vectors, recipient strains, and other tools for genetic analysis are available in Dr. Kondo's laboratory.

**Evaluation of new strains.** Fast acid producing Leuconostoc strains will be used as starters in the preparation of various fermented milk products using standard manufacturing procedures. Similarly, nisin-producing Leuconostoc will be used in Swiss cheese manufacture containing no (control) or added C. tyrobutyricum. Conventional disc assay and broth inhibition studies will be carried out to identify genetically engineered nisin-producing strains with maximum inhibitory ability against L. monocytogenes.

### **Cooperation**

This is a cooperative research project co-directed by Dr. W. E. Sandine and Dr. J. K. Kondo of the Department of Microbiology, Oregon State University and the Department of Nutrition and Food Sciences, Utah State University, respectively.

**Project Title:** Cloning the Nisin and Other Genes of Lactic Streptococci into *Leuconostoc* Species and Amplification of Nisin Production.

**Personnel:** Jeffery K. Kondo, Assistant Professor, Department of Nutrition and Food Sciences, Utah State University, Logan.

William E. Sandine, Professor, Department of Microbiology, Oregon State University, Corvallis.

Jeff Broadbent, Graduate Research Fellow, Department of Nutrition and Food Sciences, Utah State University, Logan.

Herb Wyckoff, Graduate Research Assistant, Department of Microbiology, Oregon State University, Corvallis.

Kevin Gillies, Principal Research Technician I, Department of Nutrition and Food Sciences, Utah State University, Logan.

Mary Barnes, Research Technician, Department of Microbiology, Oregon State University, Corvallis.

**Funding Sources:** Western Dairy Foods Research Center  
Utah Agricultural Experiment Station  
National Science Foundation  
United States Department of Agriculture-Agricultural Research Service  
National Needs Graduate Fellowship Program  
United States Department of Agriculture-Competitive Research Grant Program  
Oregon Agricultural Experiment Station.

### Objectives:

1. To produce and characterize lactose positive *Leuconostoc* transconjugants obtained by conjugal matings between *S. lactis* and *Leuconostoc* spp.
2. To develop transformation and gene cloning systems in *Leuconostoc*.
3. To introduce into *Leuconostoc*, plasmid-coded protease genes from lactic streptococci (e.g. *S. cremoris* Wg2).
4. To use the genetically constructed fast acid-producing *Leuconostoc* (lactose positive and protease positive) to produce different fermented dairy products such as cultured buttermilk, cottage and Gouda cheese.
5. To conjugally transfer plasmid-coded nisin genes from *S. lactis* 7962 to *Leuconostoc* and to study this plasmid at the molecular level.
6. To amplify nisin production by gene cloning techniques.
7. To use nisin producing *Leuconostoc* in Swiss cheese manufacture to inhibit gas producing anaerobic spore formers such as *Clostridium tyrobutyricum*.

8. To study the inhibition of *L. monocytogenes* by nisin and to use genetic engineering techniques to maximize its useful application.

### Results, Utah State University:

Our research for the past year has focused upon objectives 3 and 5 as described above. Studies were initiated to examine the conjugal transfer of nisin production among strains of *Lactococcus lactis* ssp. *lactis* (formerly *S. lactis*, 5). These experiments led to the development of a new, rapid and efficient method for conjugation in these organisms. Using this technique, which we term "direct-plate" conjugation (1), we have been able to more efficiently transfer the nisin production/immunity and sucrose phenotypes (4,6) into a variety of *Lactococcus lactis* ssp. *lactis* recipients (Table 1). We have also been able to transfer these traits into *Lactococcus lactis* ssp. *cremoris* SW224. Plasmid analysis confirmed the transfer of the nisin genes from *L. lactis* ssp. *lactis* 11454 to *L. lactis* ssp. *cremoris* SW224 (Figure 1). Nisin assays using the agar overlay technique described by Steele and McKay (6) demonstrated that all transconjugants produce the antibiotic (Fig. 2). We are presently involved in experiments to transfer the nisin genes into *Leuconostoc*. We plan to use other lactic acid bacteria as recipients in future experiments as we see many applications among dairy, food, and agricultural lactic fermentations.

In related research examining plasmid-encoded proteinase activity in lactococci, we have found that the proteinase genes, located on the lactose plasmid (2,3), are not sufficient for fast-acid production in many strains which acquire the plasmid. Results from conjugation and electrotransformation studies indicate that additional gene(s), located on a 43 kb plasmid of *L. lactis* ssp. *lactis* C20, are required for fast acid production in many lactococcal strains. The additional gene(s) required can be either plasmid or chromosomally encoded depending on strain. These results support prior data obtained in Dr. Sandine's laboratory where *Leuconostoc* recipients, electrotransformed with a lactococcal lactose-proteinase (Lac<sup>+</sup>Prt<sup>+</sup>) plasmid, only showed a slight increase in proteolytic activity (unpublished results). Experiments are presently underway in Dr. Sandine's laboratory to co-transform *Leuconostoc* with the Lac<sup>+</sup>Prt<sup>+</sup> and Prt factor plasmids to produce *Leuconostoc* strains with rapid-acid producing capabilities.

### Results, Oregon State University:

During the past year we have developed a successful transformation system for *Leuconostoc* species using electroporation. Various parameters such as voltage, buffer composition, and capacitance levels have been determined for obtaining optimal transformation frequencies. This system has been used to successfully transform *Leuconostoc mesenteroides* ssp. *cremoris*, *Leuconostoc mesenteroides* ssp. *dextranicum*, and *Leuconostoc lactis*. Under these conditions, transformation does not alter the resident plasmid DNA content of the recipient cells. Several cloning vectors which replicate in *Leuconostoc* have also been identified. With the electrotransformation system developed, transformation frequencies are at sufficient levels to allow for cloning directly into *Leuconostoc* spp. or to allow the transformation of intact plasmids isolated from suitable donor organisms. Without these possibilities, passage of DNA through other organisms (e.g. *Bacillus subtilis*, *Escherichia coli*) would be necessary. These situations would be of relevance when genetically engineered strains are considered for approval by appropriate regulatory agencies.

Future research involving the transformation and cloning of proteinase and lactose utilization genes from lactococci into *Leuconostoc* is now possible since the necessary components for a gene cloning system in *Leuconostoc* spp. have been defined.

### Impact of Research - Nisin:

Nisin, a polypeptide antibiotic produced by some strains of *L. lactis* ssp. *lactis*, is an effective inhibitor of gram-positive bacteria. It has been used as a food preservative in Europe since 1954 and has recently been approved for use in the United States for certain dairy products. For many years researchers have been interested in cloning the nisin genes into other organisms used to manufacture fermented foods thus enhancing the shelf-life of these products. Use of conjugation, a natural gene transfer process, is another avenue to achieve this end. New strains constructed via conjugation receive only genes that have come from safe, food-grade lactic organisms. As a result, conjugally manipulated strains will have fewer FDA restrictions than strains developed using recombinant DNA technology.

Using the direct plate conjugation method developed, we were able to transfer the nisin production/immunity and sucrose utilization genes among lactococcal strains. These results suggest that we may be able to conjugally transfer the nisin genes to other genera of lactic acid bacteria. We can envision many applications among dairy, food, and agricultural lactic fermentations for the use of nisin-producing lactic acid bacteria to enhance the shelf-life and safety of foods.

### Impact of Research - Rapid acid-producing *Leuconostoc* spp.

*Leuconostoc* spp. are slow acid-producers in milk and thus are unable to produce appreciable amounts of diacetyl in pure milk cultures. This has been accredited to the lack of proteolysis and limited lactose utilization. In mixed cultures with lactococci, the growth of *Leuconostoc* is often inhibited, resulting in the lack of desirable flavor in cultured products. It is believed that by increasing the levels of proteolysis and lactose utilization of *Leuconostoc*, flavor defects that commonly occur may be eliminated. Improved strains of *Leuconostoc* will also allow the manufacture of specialty cheeses similar to varieties now being imported or the development of new fermented dairy products. The characterization of a second proteinase factor and the development of an efficient transformation system for *Leuconostoc* now make possible the construction of *Leuconostoc* strains which grow and rapidly produce acid in milk.

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- Broadbent, J. R., Y. C. Chou, K. Gillies, and J. K. Kondo. 1989. Nisin inhibits several gram-positive, mastitis-causing pathogens. *J. Dairy Sci.* 71: in press.
- Broadbent, J. R., J. K. Kondo, and W. E. Sandine. 1989. Conjugation in *Lactococcus lactis* ssp. *lactis* on selective media. Submitted for publication, Appl. Environ. Microbiol.
- Mahmoud, H. M. A., W. E. Sandine, and J. K. Kondo. 1988. Lactose use by genetically manipulated *Leuconostoc*. *J. Dairy Sci.* 71 (Suppl. 1): 63.
- Wyckoff, H. A., W. E. Sandine, and J. K. Kondo. 1989. Electrottransformation of *Leuconostoc* with plasmid DNA. *J. Dairy Sci.* 72 (Suppl. 1):113. Manuscript in preparation.
- Kondo, J. K., and K. Gillies. 1989. Attenuation of proteinase activity results from cointegrate formation in *Lactococcus lactis* ssp. *lactis*. *J. Dairy Sci.* 72 (Suppl. 1):114.

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Kondo, J. K., H. A. Wyckoff, and W. E. Sandine. 1989. Genetic manipulation of *Leuconostoc* for improved milk fermentation. Soc. Ind. Microbiol. Ann. Meet., Seattle, WA.

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1. Broadbent, J.R., J.K. Kondo, and W.E. Sandine. 1989. Conjugation among *Lactococcus lactis* ssp. *lactis* on selective media. In submission to Appl. Environ. Microbiol. (7/89).
2. Fitzgerald, G.F., and M.J. Gasson. 1988. *In vivo* gene transfer systems and transposons. Biochimie 70:489-502.
3. Kok, J., and G. Venema. 1988. Genetics of proteinases of lactic acid bacteria. Biochimie 70:475-488.
4. Murphy, M.C., J.L. Steele, C. Daly, and L.L. McKay. 1988. Concomitant conjugal transfer of reduced-bacteriophage-sensitivity mechanisms with lactose- and sucrose-fermenting ability in lactic streptococci. Appl. Environ. Microbiol. 54:1951-1956.
5. Schleifer, K.H. 1987. Recent changes in the taxonomy of lactic acid bacteria. FEMS Microbiol. Rev. 46:201-203.
6. Steele, J.M., and L. L. McKay. 1986. Partial characterization of the genetic basis for sucrose metabolism and nisin production in *Streptococcus lactis*. Appl. Environ. Microbiol. 51:57-64.

Table 1. Conjugal transfer frequencies using direct and milk agar plate techniques.

Mating donor x recipient	Selected phenotype	Technique	Transfer frequency <sup>1</sup> transconjugants/donor
ML3 x LM2306	Lac <sup>+</sup> Ery <sup>r</sup>	Milk agar; Rp <sup>2</sup>	2.2 x 10 <sup>-9</sup>
ML3 x LM2306	Lac <sup>+</sup> Ery <sup>r</sup>	Milk agar; harvest <sup>3</sup>	1.1 x 10 <sup>-7</sup>
ML3 x LM2306	Lac <sup>+</sup> Ery <sup>r</sup>	Direct-plate	1.1 x 10 <sup>-7</sup>
ML3 filtrate x LM2306	Lac <sup>+</sup> Ery <sup>r</sup>	Direct-plate	0
ML3 + DNase x LM2306 + DNase	Lac <sup>+</sup> Ery <sup>r</sup>	Direct-plate	6.2 x 10 <sup>-8</sup>
Non-viable ML3 <sup>4</sup> x LM2306	Lac <sup>+</sup> Ery <sup>r</sup>	Direct-plate	0
ATCC 11454 x LM2306	Suc <sup>+</sup> Ery <sup>r</sup>	Milk agar; Rp <sup>2</sup>	2.4 x 10 <sup>-8</sup>
ATCC 11454 x LM2306	Suc <sup>+</sup> Ery <sup>r</sup>	Milk agar; harvest <sup>3</sup>	1.9 x 10 <sup>-6</sup>
ATCC 11454 x LM2306	Suc <sup>+</sup> Ery <sup>r</sup>	Direct-plate	1.3 x 10 <sup>-5</sup>
ATCC 11454 filtrate x LM2306	Suc <sup>+</sup> Ery <sup>r</sup>	Direct-plate	0
ATCC 11454 + DNase x LM2306 + DNase	Suc <sup>+</sup> Ery <sup>r</sup>	Direct-plate	2.7 x 10 <sup>-7</sup>
Non-viable x LM2306	Suc <sup>+</sup> Ery <sup>r</sup>	Direct-plate	0
ATCC 11454 <sup>4</sup>			

<sup>1</sup>Donor CFU was calculated using initial donor counts for direct and replica-plated milk agar plates. Frequencies for harvested milk agar plates were determined using modified donor counts (see text).

<sup>2</sup>Transconjugants obtained by replica-plating onto selective media (2).

<sup>3</sup>Conjugal mixture harvested from milk agar using 1 ml 0.85% saline and then plating cells on selective media (8).

<sup>4</sup>Donors heat-killed (55°C for 1 h) or chlorox treated (6).



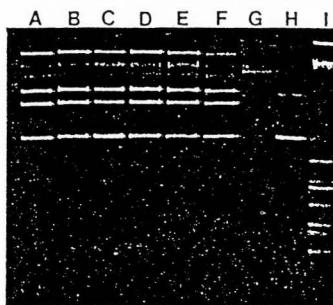


Figure 1. Plasmid profiles of *Lactococcus lactis* ssp. *cremoris* SW224 nisin-producing transconjugants. Lanes A-F, Nisin-producing SW224 transconjugants; Lane G, SW224; Lane H, JB0213 recipient strain containing pGK13; Lane I, *E. coli* V517 reference mobility plasmids.

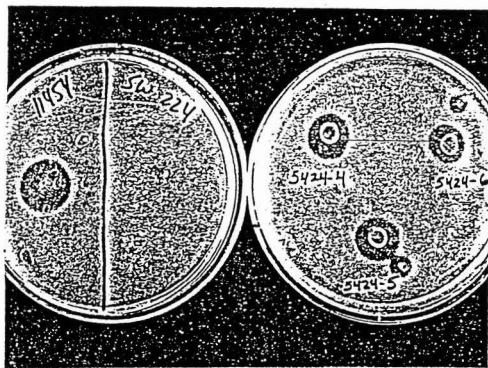


Figure 2. Nisin assays of transconjugants. Left plate, 11454 nisin-producing donor strain showing inhibition of indicator strain (*L. lactis* ssp. *lactis*)

- Project Title:** Cloning the Nisin and other Genes from Lactococcus into Leuconostoc Species and Amplification of Nisin Production.
- Personnel:** W. E. Sandine, Principal Investigator, Department of Microbiology, Oregon State University
- H. A. Wyckoff, Department of Microbiology, Oregon State University
- M. Barnes, Department of Microbiology, Oregon State University
- K. Gillies, Department of Microbiology, Oregon State University.
- Funding:** Western Dairy Foods Research Center.

### Objectives:

1. To produce and characterize lactose-positive Leuconostoc transconjugants obtained by electro-transformation between Lactococcus lactis and Leuconostoc spp .
2. To introduce into Leuconostoc, plasmid-coded protease genes from lactococci (e.g. Lactococcus cremoris Wg2).
3. To use genetically constructed fast acid-producing leuconostoc (lactose-positive and protease-positive) to produce different fermented dairy products such as cultured buttermilk, cottage and Gouda cheese.

### Results:

Previous progress reports from our laboratory have outlined a number of advances made toward the meeting of the objectives stated above. Transformation of Leuconostoc spp. of interest to the dairy industry via electroporation is now a commonplace procedure in our laboratory and allows us to easily introduce foreign DNA into Leuconostoc spp. We have recently extended these observations so that all species of Leuconostoc used in dairy fermentations have been transformed with cloning and shuttle vectors (see Wyckoff et al. 1990 below).

As the transformation procedures for Leuconostoc spp. have been worked out, we have been worked out, we have turned our attention to objectives 1 and 2 above. We noted in last year's report the preliminary observation that Leuconostoc cremoris 19254 had been transformed with Lactococcus lactis DNA. These

transformants had elevated beta-galactosidase and phospho-beta-galactosidase activities. In addition, some of these transformants were found to have elevated protease activity. To date, after numerous attempts, this has not been repeated, and the original transformants no longer behave as reported.

Given the above results, we have decided to abandon for the time being transformation with L. lactis native plasmid or chromosomal DNA and concentrate on transforming Leuconostoc spp. with genes cloned into plasmid vectors. The genes that we would like to transfer to Leuconostoc from the lactococci are lacG encoding phospho-beta-galactosidase and perhaps lacFE encoding phosphoenolpyruvate-dependent phosphotransferase system components Factor III and Enzyme II--both specific for the utilization of lactose. To this end we have cloned and expressed the lacG product in Escherichia coli on a shuttle vector pNZ18. We have shown, previously, that the vector can be transferred to Leuconostoc spp. at frequencies of ca. 10 transformants per mg of DNA. We have also shown that the chimeric pNZ18:lacG plasmid purified from E. coli can be transferred to Leuconostoc. Unfortunately, the chimeric plasmids recovered from the transformants had significant portions of the DNA deleted.

These results signal that there may be problems associated with the expression of lacG in the host strain used, since DeVos and coworkers recently reported the expression of a lacG clone in both E. coli and Leuconostoc paramesenteroides. So little is known of restriction and modification systems in the Leuconostoc that it may be that we inadvertently picked a host strain with a restriction system that recognizes the foreign DNA that we wish to introduce and degrades it. We have, however, picked strains that are important to dairy product manufactures instead of strains based on their ease of manipulation.

Presently, experiments are also underway to isolate the chromosomal genes that code for the lactose permease in Streptococcus thermophilus. Our objective is to place the beta-galactosidase gene and the lactose permease gene from S. thermophilus on a single plasmid. We will then transform Leuconostoc with this plasmid and assay for reconstitution of lactose metabolism in the host.

In the last year we have begun experiments on introducing proteinase genes into Leuconostoc spp., particularly Leuconostoc dextranicum 44-4. Initial experiments involved transformation of strain 44-4 with a prtMP clone (supplied by J. Kondo, Utah State University). This proteinase gene is not completely functional even in Lactococcus lactis LM2306, where it was originally cloned, but we have used it to give us some idea of our ability to introduce and maintain foreign DNA in strain 44-4. A recombinant plasmid containing the proteinase gene from Lactococcus lactis was found in transformants from this experiment, however, many of these also contained insertions and deletions. Again, these experiments indicate our ability to transform Leuconostoc, but also indicate that our choice of host strain has to be re-examined. We are also working to obtain a fully functional prtMP clone.

Because of work on the genetics of proteolysis in the lactococci, it is becoming increasingly clear that the presence of the lactococcal-type proteinase (prtMP product) in a strain is not enough for fast growth in milk, i.e. for the utilization of casein as a source of amino acids and nitrogen. Von Wright and co-workers in

Finland and J. Kondo and K. Gillies at Utah State University have shown that a gene block that we call PrtA is absolutely required in addition to proteinase genes for growth in milk but is not required for growth in M17-broth culture. Kondo and Gillies have further demonstrated that the PrtA block is located on pJK430, a 43 kb plasmid in Lactococcus lactis C20, and is chromosomally encoded in Lactococcus lactis C2 (Manuscript in preparation). We have demonstrated that the PrtA block is located on a 10 kb BamHI restriction fragment of pJK430 and we are currently attempting to clone this fragment in a lactococcal host. The activity of PrtA most probably is hydrolysis and/or transport of small peptides made available to the cell by the hydrolysis of caseins by the proteinase enzyme.

Genetic analysis of PrtA has been hampered in the L. lactis C20 system by the fact that the PrtA phenotype is not a good selective marker for the presence of pJK430 in otherwise plasmid-free strains. We have overcome this problem by inserting a transposon (Tn919) encoding resistance to the antibiotic tetracycline into the plasmid. This was accomplished by mating an Enterococcus faecalis strain containing Tn919 as a conjugative transposon with a streptomycin-resistant isolate of L. lactis C20. Tn919 was shown to integrate into pJK430, without loss of PrtA function, by DNA-DNA hybridization studies and the demonstration of transfer of tetracycline resistance with the transfer of the pJK430:Tn919 plasmid via electroporation.

Now that we have pJK430:Tn919 isolated in a strain, we have a source of DNA that we can use for cloning of the PrtA gene block. It may be that the PrtA block will also have to be added to Leuconostoc spp. to enable them to grow rapidly in milk, depending of course on the strains native ability to process and transport peptides.

Finally, we have begun thinking of the final form in which we would like to add foreign DNA to Leuconostoc spp. Since Leuconostoc plasmids are extremely stable in host strains, we are interested in developing cloning vectors based on a 3 kb Leuconostoc dextranicum 226 plasmid. This work is just beginning, but restriction mapping has already identified a number of restriction sites that will be useful in our efforts to construct the vector.

In the course of this work, we have modified the technique of colony hybridization, a standard a method in E. coli molecular biology, so that it can be used to identify sequence homology of a known DNA probe with that of DNA released from isolated Leuconostoc colonies on nylon membranes. This methodological advance, like others, will build up our stock of tools that are necessary for genetic engineering in these organisms. In addition, we plan to sequence the plasmid so that we might better understand how plasmid structure in the Leuconostoc compares to other more thoroughly studied plasmids in gram-positive and gram-negative bacteria. Although this work will no doubt require more time than the current funding period, basic studies like this will enable us to design experiments from a more sound conceptual base, and will perhaps allow us to make modification in the genetic complement of Leuconostoc spp. with greater ease in the future.

## Impact of Research:

While a great deal of research effort has been focused on the genetics of lactose fermentation and proteolysis in the lactococci, very little is known about the genetics of the genus Leuconostoc. The results outlined above, as well as those previously reported, help lay the groundwork for the genetic engineering of Leuconostoc spp. for the dairy industry. Fulfillment of the objectives outlined above will allow enhanced use of Leuconostoc spp. in dairy starter cultures and may make possible the production of specialty cheeses to expand the market of dairy products.

## Publications and Abstracts:

Wyckoff, H.A., W.E. Sandine and J.K. Kondo. 1989. Electroporation of Leuconostoc with plasmid DNA. J. Dairy Sci. 71:Suppl. 1 Abst.

Kondo, J.K., H.A. Wyckoff, and W.E. Sandine. 1989. Genetic manipulation of Leuconostoc for improved milk fermentations. Society for Industrial Microbiology meeting. Abst.

Wyckoff, H.A., W.E. Sandine, and J.K. Kondo. 1990. Transformation of dairy Leuconostoc using plasmid vectors from Bacillus, Escherichia coli, and Lactococci hosts. Manuscript in preparation.

- Project Title:** Cloning the Nisin and other Genes from Lactococcus into Leuconostoc Species and Amplification of Nisin Production.
- Personnel:** Jeffery K. Kondo (PI), Associate Professor,  
Department of Nutrition and food Sciences, USU.
- Jeffery R. Broadbent, Graduate Research Fellow,  
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- Funding Sources:** Western Dairy Foods Research Center  
National Science Foundation  
Utah Agricultural Experiment Station  
USDA-ARS National Needs Graduate Fellowship  
Program
- Objectives:**
1. To produce and characterize lactose positive Leuconostoc transconjugants obtained by conjugal matings between Lactococcus and Leuconostoc spp.
  2. To develop transformation and gene cloning systems in Leuconostoc.
  3. To introduce into Leuconostoc, plasmid-coded protease genes from lactococci (e.g. L. lactis ssp. cremoris Wg2).
  4. To use the genetically constructed fast acid-producing Leuconostoc (lactose positive and protease positive) to produce different fermented dairy products such as cultured buttermilk, cottage and Gouda cheese.
  5. To conjugally transfer plasmid-coded nisin genes from L. lactis ssp. lactis 7962 to Leuconostoc and to study this plasmid at the molecular level.
  6. To amplify nisin production by gene cloning techniques.
  7. To use nisin producing Leuconostoc in Swiss cheese manufacture to inhibit gas producing anaerobic sporeformers such as Clostridium tyrobutyricum.

8. To study the inhibition of L. monocytogenes by nisin and to use genetic engineering techniques to maximize its useful application.

## Results:

Our research has been focused upon objective 3 as outlined above. We have been investigating the conjugal transfer of nisin-production and immunity using a rapid new method of conjugation, called direct-plate conjugation (DPC), that we reported last year. These experiments have allowed us to accomplish several important steps. These include the development of an improved efficiency of transfer for nisin-producing capability, successful transfer of nisin-producing ability into several strains of L. lactis ssp. cremoris, as a model for transfer techniques into Leuconostoc, and the demonstration that cell surface proteins are involved in high-frequency conjugal transfer of lactose utilization capability.

The studies we have performed to maximize the efficiency of transfer for nisin producing ability have allowed us to increase the frequency 10- to 100-fold for transfer among L. lactis ssp. lactis (Table 1). This capability has permitted our subsequent studies of transfer into, and expression in, L. lactis ssp. cremoris (Table 2). We selected L. lactis ssp. cremoris as a model system for studying low frequency transfer of nisin production for eventual application to Leuconostoc spp. These investigations were not possible using traditional conjugal methods because the frequencies were usually so low as to be undetectable. Among the results of these studies was the discovery that the stability of the nisin-producing phenotype is strain dependant. This is important because the only nisin-producing Leuconostoc transconjugant obtained thus far was unstable, but data from the L. lactis ssp. cremoris experiments suggests that stable Leuconostoc hosts may be found. These experiments are presently in progress.

Studies are also in progress to investigate the ability of the nisin-producing L. lactis ssp. cremoris transconjugants to inhibit a variety of Gram-positive pathogenic and spoilage organisms, including S. aureus, L. monocytogenes, Bacillus spp., and Clostridium spp. In results relating to objective 6, data for these transconjugants shows that some are able to produce more nisin than the L. lactis ssp. lactis donor strain, 11454. In addition, we have discovered that these transconjugants also acquired genes for reduced sensitivity to some bacteriophage and that the ability to produce acid in milk is not affected by the nisin-producing phenotype (Table 3). These results suggest nisin-producing L. lactis ssp. cremoris may be well suited to industrial use and our studies aimed at this application will continue.

Additional experiments using DPC have shown cell surface proteins are involved in high-frequency conjugal transfer of lactose utilization. Follow up studies are in progress to identify and characterize these proteins and

determine whether they can be introduced into other cells to increase the transfer frequency of other traits, such as nisin producing ability.

### Impact of Research:

Nisin is a peptide antibiotic produced by some strains of Lactococcus lactis ssp. lactis. It inhibits a variety of Gram-positive organisms and evidence has now been presented indicating some Gram-negatives may also be affected. For many years researchers have been interested in cloning the nisin genes into other organisms used to manufacture fermented foods to enhance the safety and shelf stability of these foods.

Studies of the genetic basis for nisin production have demonstrated that nisin is synthesized as a precursor protein that requires post-translational modification to yield the active antibiotic. Although the nisin precursor gene has been cloned and sequenced, nisin production by clones has not been reported. Production will likely require cloning of the genes encoding the enzymes needed for activation and, if the cloning host is sensitive, genes for nisin immunity. The use of conjugation, a natural form of gene transfer, is a simpler and well established way to introduce nisin producing ability into other organisms.

Because strains constructed via conjugation receive only DNA from safe, food grade lactic organisms, they will have fewer FDA restrictions than strains constructed using recombinant DNA technology. This approach is very attractive because many important traits in dairy strains are conjugative, including nisin production, lactose and protease utilization, and phage resistance. The development of direct plate conjugation has allowed us to overcome some of the limitations present in traditional methods for conjugation and construct several strains of nisin-producing L. lactis ssp. cremoris as models for application to Leuconostoc. Results of these experiments have suggested that stability in Leuconostoc may also be strain dependent and therefore stable hosts may be obtained.

In addition, our studies suggest it is possible to construct nisin-producing L. lactis ssp. cremoris from industrial strains to use as blends in mixed and multiple starter systems. Studies are underway to evaluate the possible safety and preservative benefits these strains may provide. Finally, our experiments aimed at identifying the cell surface proteins involved in high-frequency conjugation may eventually allow for an even broader application of conjugation to dairy strain improvement.

### Publications:

Broadbent, J.R., Y.C. Chou, K. Gillies, and J.K. Kondo. 1989. Nisin inhibits several gram-positive, mastitis-causing pathogens. J. Dairy Sci. 72:3342-3345.



- Broadbent, J.R., and J.K. Kondo. 1990. Genetic construction of nisin-producing L. lactis spp. cremoris and analysis of a rapid method for conjugation. Manuscript submitted to Appl. Environ. Microbiol.

#### Abstracts:

- Broadbent, J.R., and J.K. Kondo. 1990. Genetic construction of nisin-producing strains of Lactococcus lactis ssp. cremoris. J. Dairy Sci. 73 (Suppl. 1):72.
- Gillies, K., and J.K. Kondo. 1990. Plasmid encoded elements required for proteolysis of milk proteins by Lactococcus lactis ssp. lactis. Third symposium on lactic acid bacteria; genetics, metabolism, and applications, Wageningen, The Netherlands.

Table 1. Comparison of conjugal transfer frequencies using DPC and milk agar plate techniques.

<u>Mating</u> donor x recipient	Selected phenotype	Technique	<u>Transfer frequency</u> transconjugants/donor CFU
ML3 x LM2306	Lac+Em <sup>r</sup>	Milk agar; RP <sup>a</sup>	2.2 x 10 <sup>-9</sup>
ML3 x LM2306	Lac+Em <sup>r</sup>	Milk agar; harvest <sup>b</sup>	1.1 x 10 <sup>-7</sup>
ML3 x LM2306	Lac+Em <sup>r</sup>	DPC	1.1 x 10 <sup>-7</sup>
ML3 filtrate x LM2306	Lac+Em <sup>r</sup>	DPC	0
ML3+DNase x LM2306+DNase	Lac+Em <sup>r</sup>	DPC	6.2 x 10 <sup>-8</sup>
Non-viable ML3 <sup>c</sup> x LM2306	Lac+Em <sup>r</sup>	DPC	0
ATCC 11454 x LM2306	Suc+Em <sup>r</sup>	Milk agar; RP <sup>a</sup>	2.4 x 10 <sup>-8</sup>
ATCC 11454 x LM2306	Suc+Em <sup>r</sup>	Milk agar; harvest <sup>b</sup>	1.6 x 10 <sup>-6</sup>
ATCC 11454 x LM2306	Suc+Em <sup>r</sup>	DPC	7.0 x 10 <sup>-6</sup>
ATCC 11454 filtrate x LM2306	Suc+Em <sup>r</sup>	DPC	0
ATCC 11454+DNase x LM2306+DNase	Suc+Em <sup>r</sup>	DPC	8.7 x 10 <sup>-7</sup>
Non-viable x LM2306 ATCC 11454 <sup>c</sup>	Suc+Em <sup>r</sup>	DPC	0

<sup>a</sup>Transconjugants obtained by replica-plating onto selective media (2).

<sup>b</sup>Conjugal mixture harvested from milk agar using 1 ml 0.85% saline and plating cells on selective media (27).

Table 2. Frequency of Nip+Suc+ transfer into *L. lactis* ssp. *cremoris* using DPC.

<b><u>Direct-plate conjugation of Nip+Suc+</u></b>	
Recipient	Nip+Suc+ Transfer frequency (Transconjugants/donor CFU)
<i>L. lactis</i> ssp. <i>cremoris</i> SW 224 (Protoplast transformed with pGB301; Woskiew and Kondo, 1987)	$3.6 \times 10^{-7}$
<i>L. lactis</i> ssp. <i>cremoris</i> JKEB30 (Electroporated with pGB301)	$4.5 \times 10^{-8}$
<i>L. lactis</i> ssp. <i>cremoris</i> JBC313 (Electroporated with pGK13)	$3.9 \times 10^{-7}$

Table 3. Characteristics of Nip+Suc+ transconjugants.

Strain	Sensitivity to O <sub>7962</sub>	Fast acid production
<b><u>L. lactis ssp. lactis:</u></b>		
1145-4 (Nip+Suc+ donor)	-	not determined
<b><u>L. lactis ssp. cremoris:</u></b>		
CS2/24	+	+
JB54/24 (Nip+Suc+)	-	+
EB7	+	+
JB54/EB (Nip+Suc+)	-	+
C3	+	+
JB54/C3 (Nip+Suc+)	-	+

**Project Title:** Cloning the Nisin and other Genes from *Lactococcus* Into *Leuconostoc* Species and Amplification of Nisin Production

**Personnel:** Jeffery K. Kondo, Associate Professor, Dept. of Nutrition & Food Sciences, Utah State University, Logan.

William E. Sandine, Professor, Department of Microbiology, Oregon State University, Corvallis.

Jeffery R. Broadbent, Graduate Research Fellow, Dept. NFS, USU.

Herb Wycoff, Graduate Research Assistant, Dept. Microbiology, OSU.

Hua Wang, Graduate Research Assistant, Dept. NFS, USU.

Yat-Chen Chou, Graduate Research Assistant, Dept. NFS, USU.

**Funding:** Western Dairy Foods Research Center  
National Science Foundation  
Utah Agricultural Experiment Station  
USDA-ARS National Needs Graduate Fellowship Program

**Objectives:**

1. To produce and characterize lactose positive *Leuconostoc* transconjugants obtained by conjugal matings between *Lactococcus* and *Leuconostoc* spp.
2. To develop transformation and gene cloning systems in *Leuconostoc*.
3. To introduce into *Leuconostoc*, plasmid-coded protease genes from lactococci (e.g. *L. lactis* ssp. *cremoris* Wg2).
4. To use the genetically constructed fast acid-producing *Leuconostoc* (lactose positive and protease positive) to produce different fermented dairy products such as cultured buttermilk, cottage and Gouda cheese.
5. To conjugally transfer plasmid-coded nisin genes from *L. lactis* ssp. *lactis* 7962 to *Leuconostoc* and to study this plasmid at the molecular level.
6. To amplify nisin production by gene cloning techniques.
7. To use nisin producing *Leuconostoc* in Swiss cheese manufacture to inhibit gas producing anaerobic sporeformers such as *Clostridium tyrobutyricum*.

83. To study the inhibition of *L. monocytogenes* by nisin and to use genetic engineering techniques to maximize its useful application.

## Results, Utah State University:

Our research has been focused upon objectives 3 and 6 as outlined above. We have continued our investigation of conjugal transfer of nisin production and immunity in a lactococcal model system. These studies have examined the effects of various physiological and environmental factors upon transfer efficiency, in order to develop methodology which will permit transfer into *Leuconostoc* sp. Results from these experiments have allowed us to accomplish several important steps. First, as reported last year, was the development of the direct plate conjugation technique (DPC) which improved the efficiency of transfer for nisin-producing capability. This led to the successful transfer of nisin-producing ability into several strains of *L. lactis* ssp. *cremoris*. During the past year we were able to conjugally transfer the nisin genes into 3 strains of *Streptococcus salivarius* ssp. *thermophilus*. Transconjugants of these strains were immune to nisin and also acquired sucrose fermenting ability, but nisin production was not detected by an agar overlay assay. Hybridization data, however, indicated that the nisin structural gene, *nisA*, was present in transconjugants. Our investigations have also suggested that the conjugative enterococcal plasmid pAM-beta-1 (5) facilitated the intergeneric transfer. Experiments are presently underway to determine if the use of pAM-beta-1 will also facilitate transfer of these genes to other dairy microorganisms such as *Leuconostoc* sp.

In related research, we used *Escherichia coli* to clone a 10 kb *Kpn* I fragment which contained the *nisA* gene from the nisin-producing strain *Lactococcus lactis* ssp. *lactis* 11454. Sequence data published by other investigators (1,2) indicated that the *nisA* gene was located approximately 1.5 kb downstream of the 5' end of our fragment and this orientation was supported by restriction analysis of the fragment. Published data also showed that our fragment lacked an RNA polymerase promoter sequence for transcription of *nisA* and one subsequent gene which are part of a polycistronic operon (4). Because genetics studies have indicated that the genes for nisin production and immunity, sucrose utilization, and reduced bacteriophage sensitivity are linked (3), we wished to determine whether our fragment would express one or more of these phenotypes in a lactococcal host. To accomplish this we constructed plasmid pJB100, which placed the fragment downstream of the promoter for the erythromycin resistance gene of the vector pGK13. After *Lactococcus lactis* ssp. *lactis* LM0230 was electrotransformed with pJB100, however, we were not able to detect any changes in the phenotypes of transformants. Additional studies are underway to determine whether the presence of pJB100 in nisin-producing lactococcal transconjugants may alter levels of nisin production through a gene dosage effect.

## Impact of Research - Nisin:

Nisin, a peptide antibiotic produced by some strains of *Lactococcus lactis* ssp. *lactis*, is an effective inhibitor of Gram-positive bacteria. The antibiotic has been approved for use as a food preservative in over forty-five countries, including the United States. For

many years, investigators have been interested in transferring the nisin genes into other organisms used to manufacture fermented foods to enhance the shelf life of these products. Conjugation, a natural gene transfer process, may be useful to achieve this goal. Bacterial strains that are developed via conjugation contain genes that only come from other safe, food-grade lactic acid bacteria. Consequently, conjugally improved strains may face fewer FDA restrictions, with respect to industrial application, than strains which are improved through recombinant DNA technology.

Our studies of conjugation have yielded new methodology which has allowed us to investigate many of the parameters which affect transfer of the nisin genes. This work has led to improved transfer efficiency and the subsequent ability to transfer these genes into strains of *Lactococcus lactis* ssp. *cremoris* and *Streptococcus salivarius* ssp. *thermophilus*. These results indicate that we may be able to use conjugation to introduce the nisin genes into leuconostocs and other genera of lactic acid bacteria. We can foresee many applications for nisin-producing lactic organisms among dairy, food, and agricultural fermentations, to enhance the safety and shelf-life of foods.

### **Construction of rapid acid-producing leuconostocs:**

*Leuconostoc* ssp. are slow acid-producers in milk and thus are unable to produce appreciable amounts of the important flavor compound, diacetyl, in pure milk cultures. This shortcoming has been attributed to the lack of milk proteolysis and limited lactose utilization. If the growth of leuconostocs is slow or inhibited, the resulting product lacks desirable flavor. Increasing the levels of proteolysis and lactose utilization, by gene transfer from lactococci to leuconostocs, may reduce or eliminate flavor defects in fermented milks. Improved *Leuconostoc* ssp. may also allow the manufacture of specialty cheese similar to varieties now imported, or allow development of novel dairy products.

### **Publications:**

Broadbent, J.R., and J.K. Kondo. 1990. Genetic construction of nisin-producing *L. lactis* ssp. *cremoris* and analysis of a rapid method for conjugation. Appl. Environ. Microbiol. 57:517-524.

### **Abstracts:**

Broadbent, J.R., and J.K. Kondo. 1990. Genetic construction of nisin-producing strains of *Lactococcus lactis* ssp. *cremoris*. J. Dairy Sci. 73 (Suppl. 1):72.

Wang, H., and J.K. Kondo. 1991. Study of the physical and functional characteristics of cell clumping in Lac<sup>+</sup> transconjugants of *Lactococcus lactis* ssp. *lactis* ML3. J. Dairy Sci. 74 (Suppl. 1):25.

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- Stein, M.T., Y.J. Chung, and J.N. Hansen. Characterization of the nisin gene as part of a polycistronic operon in the chromosome of *Lactococcus lactis* 11454. *Appl. Environ. Microbiol.* 57:1181-1188.
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Project Title: Cloning the Nisin and Other Genes of Lactic Streptococci into *Leuconostoc* Species and Amplification of Nisin Production

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Funding Sources: Western Dairy Foods Research Center

Objectives:

This has been a joint project between USU and OSU. Research on nisin has been done at USU while development of *Leuconostoc* - *Lactococcus* genetic exchange procedures has been done at OSU. *Leuconostoc* bacteria are mixed with *Lactococcus* strains to produce and market different types of milk products. The *Leuconostoc* produce flavor (diacetyl) while the lactococci produce acid. Both are required and neither can produce what the other produces. Therefore in this research we are developing methods to introduce lactococcal genes into *Leuconostoc* in order that the latter can be used alone to produce traditional and innovative fermented dairy products.

1. To produce and characterize lactose positive *Leuconostoc* transconjugants obtained by conjugal matings between *Lactococcus lactis* and *Leuconostoc* spp.

2. To develop transformation and gene cloning systems in *Leuconostoc*.

3. To introduce into *Leuconostoc*, plasmid-coded-protease genes from lactic streptococci.

4. To use the genetically constructed fast acid-producing *Leuconostoc* to produce different fermented dairy products such as cultured buttermilk, cottage and Gouda cheese.

5. To conjugally transfer plasmid-coded nisin genes from *Lactococcus lactis* to *Leuconostoc*.

6. To amplify nisin production by gene cloning techniques.

7. To use nisin producing *Leuconostoc* in Swiss cheese manufacture to inhibit gas producing anaerobic spore formers such as *Clostridium tyrobutyricum*.

8. To study the inhibition of *L. monocytogenes* by nisin and to use genetic engineering to maximize its useful application

Results :

In our previous yearly reports, we have discussed the progress that we have made towards fulfilling the objectives of our studies on *Leuconostoc*. In summary, we have developed a genetic transformation system which has been applicable to all species of dairy *Leuconostoc*. By using this procedure, we have identified a number of cloning vectors which can be used in genetic studies pertaining to *Leuconostoc*. Although unsuccessful, preliminary attempts to express selected lactococcal genes in *Leuconostoc* have been made. In addition, a study dealing with the

on nisin inhibition of Listeria monocytogenes has been completed and published.

In the past year, we have focused primarily on the characterization of a small cryptic plasmid in Leuconostoc dextranicum 226 designated pMBB1. Our goal in this regard is two fold. One, we want to gain some basic information about plasmid replication and maintenance in Leuconostoc. Some of the industrially important plasmids in lactic streptococci, (lactose utilization and proteolysis) plasmids in Leuconostoc are very stable. Secondly, we plan to use this plasmid as a backbone for construction of cloning vectors which will be based on a Leuconostoc replicon which presumably have the same stability as native Leuconostoc plasmids.

Specifically, we have mapped pMBB1 with restriction endonucleases and sized it at approximately 2.85 kilobases in length. By using molecular cloning techniques, we have located the origin of replication on a 1.9 Kb MboI fragment. We are currently sequencing pMBB1 and to have the complete nucleotide sequence of the 1.9Kb fragment containing the origin of replication as well as most of the remaining sequence. Efforts are under way to determine the homology of pMBB1 to other plasmids, possible open reading frames, and other characteristics by comparing the sequence to known sequences in computer databanks. An in vitro prokaryotic transcription-translation assay has allowed us to identify two proteins that are encoded by pMBB1. In addition, we have cloned a chloramphenicol resistance gene onto the 1.9 Kb fragment, completing the first step towards constructing a cloning vector. This construction has allowed us to determine that this plasmid has the ability to replicate in Lactococcus lactis as well as Leuconostoc species other than Leuconostoc dextranicum.

#### Impact of Research:

The results that we have produced this last year augment those that we have achieved in the previous years. By increasing our understanding of the basic molecular mechanisms of the Leuconostoc spp. we increase our chances of being able to successfully engineer these organisms with the traits that the dairy industry deems beneficial. By improving or altering the characteristics of Leuconostoc, current dairy fermentations can be improved or stabilized as well as allowing the development of new specialty dairy products or cheeses.

#### Publications:

- W. E. Sandine, H. M. A., W. E. Sandine and J. K. Kondo. 1988. Lactose use by genetically manipulated Leuconostoc. J. Dairy Science. 71:Suppl 1 Abst D-4
- W. E. Sandine, N. and W. E. Sandine. 1988. Inhibitory action of nisin against Listeria monocytogenes. J. Dairy Science. 71:3237-3245
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**Project Title:** Characterization of Bacteriophage Receptor Sites of *Lactococcus Bacteria*

**Personnel:** Bruce L. Geller, Assistant Professor, Dept. of Microbiology, Oregon State University

William E. Sandine, Professor, Dept. of Microbiology, Oregon State University

Rudy Valyasevi, Graduate Student, Dept. of Microbiology, Oregon State University.

**Funding Sources:** Western Dairy Foods Research Center  
Oregon Agricultural Experiment Station

### Objectives:

The overall objective is to understand the molecular mechanism of phage adsorption. Specifically, the objectives are:

1. To identify the bacterial component, cell wall versus cell membrane, responsible for receptor activity.
2. To define the phage receptor at the molecular level.
3. To better understand the mechanism of resistance to binding and use this information in combination with other mechanisms of resistance ultimately to produce permanently altered strains unable to be attacked by bacteriophages

### Results:

In our last progress report (1987-1988), we have shown that the receptor sites of all phages studied were located on the cell wall rather than the cell membrane. The bacterial hosts used were *L. lactis* C2 and ML3, *L. cremoris* KH and 205 and *L. diacylactis* 18-16. Subsequently we have found that the group-specific carbohydrate is the phage receptor for all of the 21 phages to 5 hosts that we have studied. We are currently collecting and analyzing data that will more narrowly define the molecular determinant of the receptor in *Lc cremoris* KH. Our strategies in pursuing this goal are: 1. to analyze and identify the cell wall components in *Lc cremoris* KH; 2. to isolate phage binding resistant mutants and determine the changes in their cell wall components from the wild type; 3. to define the determinant of binding using lectins with different specificities; 4. to study the interaction of phage to the determinant.

The cell wall of *L cremoris* KH was analyzed by Gas Chromatography (GC) and Mass Spectrometry (MS). The components found on the cell wall were rhamnose, galactose, glucose, N-acetylglucosamine and muramic acid (fig. 1 and 2). The ratio of rhamnose, galactose and glucose per unit dry weight of cell wall is approximately 6.8:1:1.6 respectively. As a first step to define the determinant of phage binding, the resistant mutants were isolated and cells with mutations in the phage binding were screened using four different phages infective to *L. cremoris* KH. The cell walls of three selected resistant mutants were analyzed by GC. In the resistant mutant 1 (RMKH 1), reductions in percentages of binding of all four phages were found (table

1). RMKH 1 had 38% less galactose than the wild type (table 2). This suggested that galactose was an important component in phage binding. The RMKH 2 cells exhibited a total loss in binding to both phages kh and ml3 and a small reduction in binding to phage 643 (table 1). RMKH 2 had 57% less rhamnose and 46% less galactose molecules than the wild type (table 2). This suggested that in addition to galactose, rhamnose was an important component in the binding of phages kh and ml 3. In RMKH 3 cells, the cell wall was found to contain 86 % less galactose, 32% more glucose and 17% more rhamnose molecules than the wild type ( table 2). An 86% loss of galactose in the cell wall of RMKH 3 contributed to a significant loss in binding of all four phages (table 1). Therefore the data suggest that galactose is essential to the binding of all four phages and both the rhamnose and the galactose are necessary for the binding of phages kh and ml 3. However it is difficult to judge from these results the specificity of binding of each of this phage.

Further evidence of the importance of each of these components to the binding of phage kh was obtained from lectin binding studies. Lectin from *Momordica charantia* (specific for galactose) inhibited the binding of phage kh to the cell wall whereas lectin from *Vicia faba* (specific to glucose) did not (fig. 3). The addition of soluble galactose to the binding assay reversed the *M. charantia* lectin inhibition of phage binding to the cell wall (fig. 4). This indicated that the lectin inhibition of binding of phage kh to the cell wall was a result of the binding of *M. charantia* lectin to galactose on the cell wall.

Although the lectin studies suggested that phage kh bound to the galactose on the cell wall, incubation of phage with D (+) galactose was not inhibitory to the phage infection (fig. 5). However, phage kh binding was inhibited up to 99 % when the phage was incubated with 0.6 M  $\alpha$ -IL (+) rhamnose. The inhibition was specific because D (+) glucose and both  $\alpha$ -D (+) and  $\alpha$ -L (-) fucoses, the stereoisomers of rhamnose, were not inhibitory (fig. 6). Further evidence that rhamnose is inhibitory to phage kh is that cells of *L. cremoris* KH grown in the presence of 0.4 M rhamnose were protected from lysis whereas cells grown in the presence of 0.1 M rhamnose or without rhamnose were lysed by phage kh (fig. 7). When cells grown in the presence of 0.4 M rhamnose were washed free from rhamnose and resuspended in M17 broth in the presence of phage, lysis of cells occurred (fig. 8). This indicates that protection of the bacterial host cells is due to phage inhibition by rhamnose rather than the emergence of a phage resistant mutant. All of these suggest that the major determinant of binding for phage kh is rhamnose and the configuration of the receptor molecule is important to phage binding

Preliminary evidence (not shown) indicates that antisera directed against the oligo-rhamnose group B specific carbohydrate from *Streptococcus pyogenes* inhibits phage kh binding to *L. cremoris* KH. This suggests that rhamnose is an important determinant of the phage kh receptor.

Galactose plays an important role in determining the phage receptor. This is apparent from the loss of galactose in all mutants analyzed, even though rhamnose was fully present in at least one of the mutants. Rhamnose also plays a crucial role in phage binding, as shown by the ability of soluble rhamnose to prevent phage infection, and the difference in loss of phage kh and ml 3 binding between RMKH 1 and RMKH 2. We speculate that galactose may determine the conformation of an oligo-rhamnose phage binding site. Alternatively, both carbohydrates may be necessary for an optimal receptor.

## Impact of Research

Bacteriophage adsorption to the bacterial host cell is the first step of infection. If phage cannot bind to its host, it cannot infect the cell. Our findings are particularly significant in that; phage binding to the cell wall fragment (non viable cells) is practically irreversible and rhamnose, the determinant of phage kh binding, is inhibitory to phage infection. Addition of a soluble receptor,

i.e., rhamnose, to a growing culture in the presence of phage irreversibly inhibits phage infection. This indicates that phages can be inactivated using mimic receptors. A strategy using mimic receptors and possibly in combination with other mechanisms of resistance needs further investigations to develop into a comprehensive phage prevention program .

#### **Publications:**

Vialyasevi, R., B. L. Geller, and W. E. Sandine. 1989. Characterization of bacteriophage receptor sites for lactococci. *J. Dairy Sci.* 72 (Suppl. 1):145.

Fig. 1 Gas Chromatography of Cell Wall components of *Lc cremoris* KH

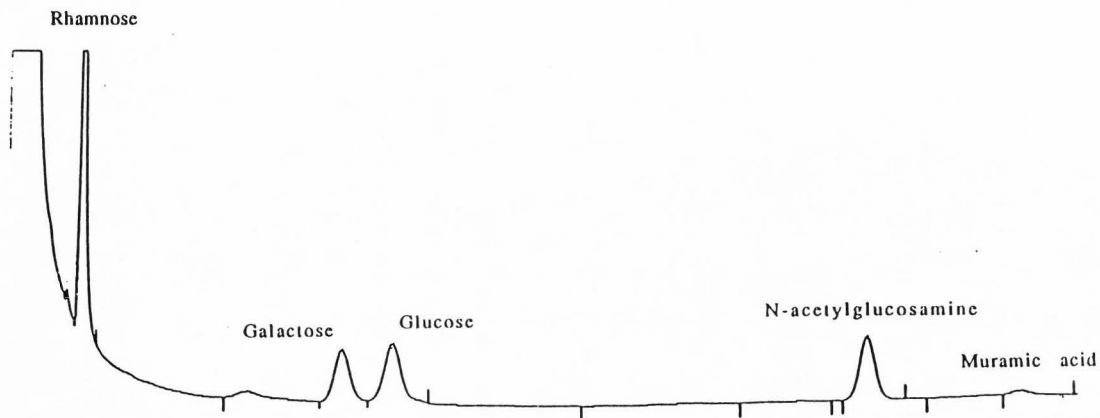
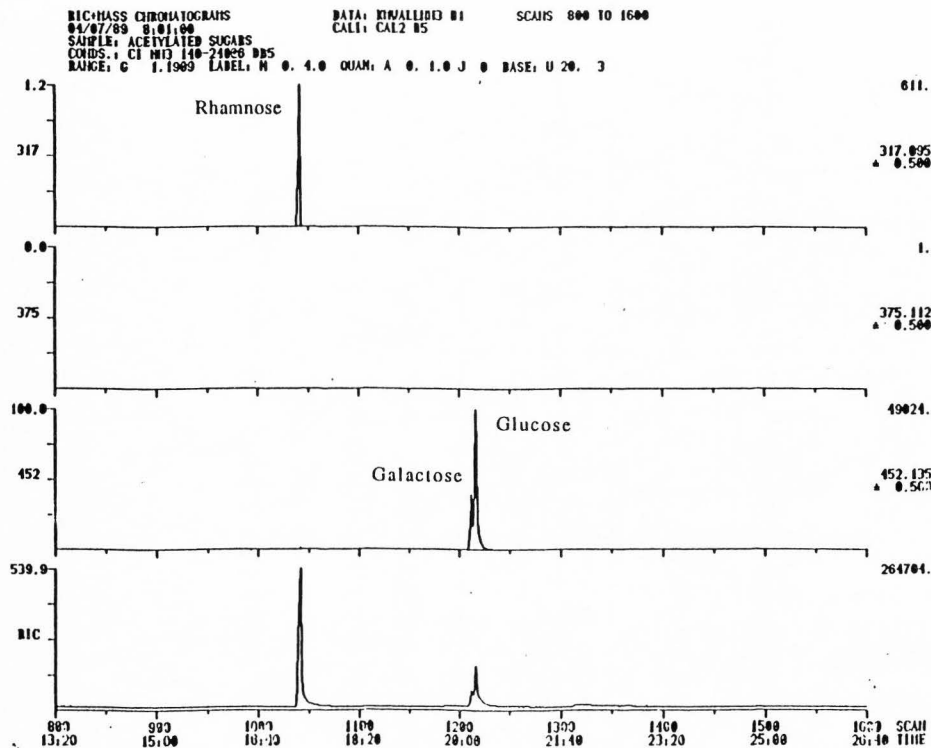


Fig. 2 Mass Spectrum Identification of Cell Wall Components of *Lc cremoris* KH.



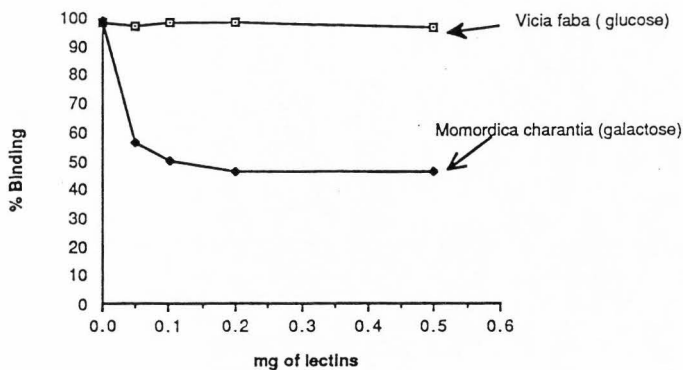


Fig. 3 Inhibition of Phage kh Binding by Lectins from *Vicia faba* (glucose) and *Momordica charantia* (galactose).

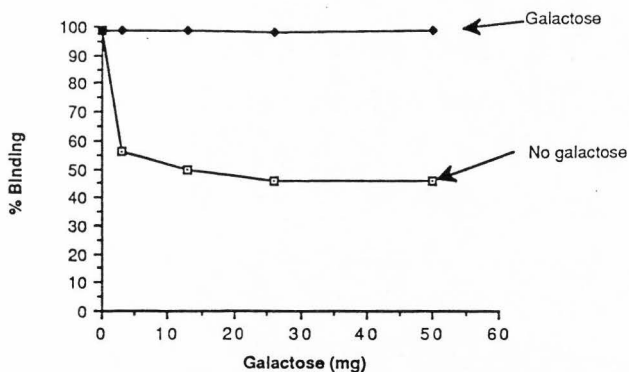


Fig. 4 Reverse of *M. charantia* Inhibition to Phage kh Binding by Galactose Molecules.



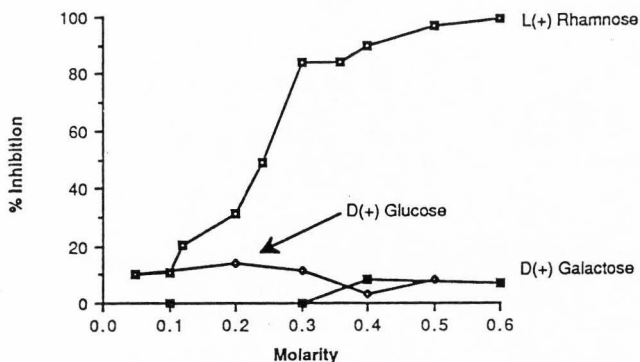


Fig. 5 Inhibition of Phage by L(+) Rhamnose, D(+) Galactose and D(+) Glucose.

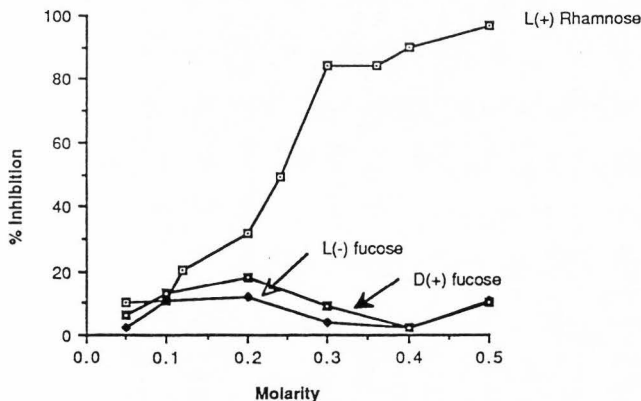


Fig. 6 Inhibition of Phage kh by L(+) Rhamnose, L(-) Fucose, and D(+) Fucose.

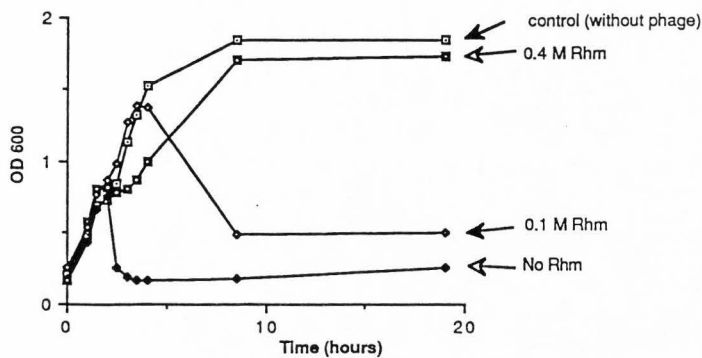


Fig. 7 Inhibition of Phage kh Infection by Rhamnose in Culture.

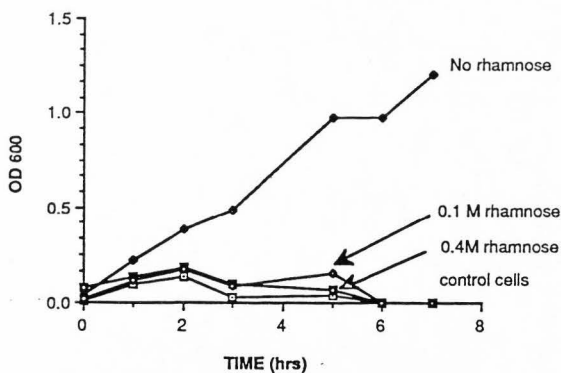


Fig. 8 Phage kh Infection of Resuspended Cells

Table 1. The Percentages of Phage Binding to Cell Walls of *Lc cremoris* KH and the Resistant Mutants.

Phage	Wall(mg)	KHWT	RMKH 1 % Binding	RMKH2	RMKH3
khkh	0.5	99-98	68-65	12-2	0
643	5	84	71-40	75-66	16-0
m13	0.5	98	71-69	0	37-0
c2	10	84	41-40	96-95	38-0

Table 2. The Concentrations of Hexose Molecules in the Cell Walls of *Lc cremoris* KH Wild Type and the Phage Resistant Mutants and Their Percentage Changes Over the Wild Type.

	Rhamnose		Galactose		Glucose	
	µg *	% change	µg *	% change	µg *	% change
KH WT	159		24		38	
RM KH 1	138	-13	15	-38	41	+8
RM KH 2	68	-57	13	-46	34	-10
RM KH 3	186	+17	3.3	-86	50	+32

+ increase

- reduction

\* expressed in equal dry weight of cell wall hydrolyzed

**Project Title:** Characterization of Bacteriophage Receptor Sites of Lactococcus Bacteria

**Personnel:** B. L. Geller, Assistant Professor, Dept. of Microbiology, Oregon State University

W. E. Sandine, Professor, Dept. of Microbiology, Oregon State University

R. Valyasevi, Graduate Student, Dept. of Microbiology, Oregon State University

**Funding Sources:** Western Dairy Foods Research Center  
Oregon Agricultural Experiment Station

### Objectives:

The overall objective is to understand the molecular mechanism of phage adsorption by lactococcus bacteria. Specifically, the objectives are:

1. To identify the bacterial components including the cell wall and the cell membrane, responsible for phage attachment, release and penetration of phage DNA.
2. To define the phage receptor at the molecular level.
3. To better understand the mechanisms of resistance of binding, release and penetration of phage DNA and use this information in combination with other mechanisms of resistance to ultimately produce permanently altered strains unable to be attacked by bacteriophages.

### Results:

In our last report, we showed that the rhamnose component of the extracellular polysaccharide of L. lactis subsp. cremoris KH is the determinant of bacteriophage kh binding. The galactose molecule of the cell wall polysaccharide also plays an indirect role in the binding and is an essential structural component of the determinant of binding. Currently we are studying the mechanism of phage adsorption to L. lactis subsp. lactis C2.

Our strategy in studying the determinant of phage binding of L. lactis subsp. lactis C2 is similar to that used with L. lactis subsp. cremoris KH. First, cells with mutations in binding were isolated and changes in the saccharide components of the cell wall polysaccharides were analyzed by gas chromatography. Lectins

with different specificities to the saccharide components of the cell wall polysaccharide were incubated with the cell walls and losses in the abilities of phage binding to the cell wall were analyzed. Lastly, we used the free saccharide receptor to compete with the cells to bind the phage and thereby inhibited the phage from lysing the cells.

The cell wall polysaccharide of *L. lactis* subsp. *lactis* C2 was analyzed by gas chromatography and found to contain rhamnose, galactose, glucose and N-acetylglucosamine. The mass ratios of rhamnose:galactose:glucose:N-acetylglucosamine were 5.3:1:1.7:2.0.

The phage resistant mutant cells were isolated by superinfecting cells with phage skl or c2. Of the approximately 50 mutants isolated from cells superinfected with phage c2, all of the cells retained their abilities to bind phage. When 8 of these mutants were tested for their abilities to form plaques, all did not form plaques. In a similar finding, any 3 of 35 phage skl resistant mutants lost their abilities to bind phage but not form plaques. These results are in contrast with our studies of the resistant mechanism of the mutant cells of *L. lactis* subsp. *cremoris* KH. Of the approximately 20 phage resistant mutants of *L. lactis* subsp. *cremoris* KH isolated from cells superinfected with phage kh, all exhibited losses in the abilities to bind phage. The results thus far suggest that the mechanism of phage resistance in most of the mutants of *L. lactis* subsp. *lactis* C2 does not involve changes in the carbohydrate receptors. It is possible that some of these mutants have altered membrane proteins which prevent the release and penetration of phage DNA.

The extracellular polysaccharides from the phage skl resistant mutants (RMSK1) were analyzed by gas chromatography. RMSK1-I showed a 149% increase in rhamnose and 47.2% decrease in glucose (table 1). These changes in the extracellular polysaccharides can be correlated with 57% and 82% reductions in binding of phages c2 and skl respectively (table 1). RMSK1-2 had an increase of 149% in rhamnose and a reduction of 23.4% in glucose in the cell wall polysaccharides which correlated with a 30% loss in binding of phage c2 and 52% of phage skl (table 1). RMSK1-3 had a 75.7% gain in rhamnose and a 32.5% reduction in glucose. These changes correlated with a 14% loss in binding of phage c2 and 17% of phage skl (table 1).

The data suggest that the losses in glucose molecules in the cell wall polysaccharides of the mutant cells can be directly correlated with the losses of phage binding. This direct correlation can be derived as follows. There are approximately the same amount of rhamnose molecules in RMSK1-I as in RMSK1-2 cells, but RMSK1-2 have 31% more glucose molecules than RMSK1-I. This higher amount of glucose molecules in RMSK1-2 cell wall polysaccharide reflects the higher percentages of phage binding to RMSK1-2 than to RMSK1-I. In addition, an increase in rhamnose in the mutants correlates with reductions in phage binding. There is more glucose in the cell wall polysaccharides of the RMSK1-2 than RMSK1-I, yet the percentages of binding of phages skl and c2 to RMSK1-2 were much lower than to RMSK3 (table 1). The increased rhamnose may mask the receptor causing a decrease in phage binding to the cell surface.

Preincubation of cell wall with lectin from Concanavalin A (lectin specific to glucose inhibited up to 40% of phage sk1 binding to the cell wall (fig. 1). This inhibition was reversed by adding glucose (fig. 2) The inhibition was specific to glucose because addition of galactose did not have the same effect (fig. 2). This suggests that Concanavalin A lectin inhibited phage sk1 binding to the receptor. In a similar experiment, lectin from Vicia faba which also binds specifically to the glucose molecule, inhibited up to 20% of phage binding. The differences in the percentages of inhibition can be explained by the difference in their molecular sizes. Lectin from V. faba (MW ~  $50 \times 10^3$ ) is one-half the molecular size of lectin from Concanavalin A (MW ~  $102 \times 10^3$ ). The reason that lectin from V. faba was not as inhibitory as lectin from Concanavalin A, is likely due to its smaller molecular mass. Other lectins specific to N-acetylglucosamine or galactose did not inhibit phage binding (data not shown).

Incubation of phage sk1 with 0.5M rhamnose decreased phage binding by 99% (fig. 3). This inhibition is specific to rhamnose as other sugar found on the cell wall (glucose, galactose, glucosamine or N-acetylglucosamine) did not inhibit (fig. 3). This seems to suggest that the primary receptor for phage sk1 is the rhamnose component of the extracellular polysaccharide. However when 0.6M rhamnose was included in the growing culture in the presence of phage sk1, cells were not completely protected from lysis by the phage (fig. 4). After about 6 hours, cells growing in the presence of rhamnose and phage started to lyse. The rate of cell lysis was noticeably slower than the control cultures that did not contain any rhamnose molecules. This was in contrast to phage c2 infection, where 0.6M rhamnose prevented lysis (fig. 4).

The preliminary data suggest that the molecular components of phage sk1 receptor may be a combination of rhamnose and glucose. The mechanism of resistance to phage c2 and phage sk1 in most of the resistant cells appears to be mutations at steps subsequent to the initial attachment of the cell surface receptor. Mutations in membrane proteins which caused blockage of the release and penetration of phage DNA have been reported in phage T4 and lambda phage. Moreover, in a closely related strain of L. lactis subsp. lactis C2, phage m13 has been reported to be inactivated by the lipoproteins from the cell membrane of L. lactis subsp. lactis ML3. It is possible that the resistance mechanism of the mutant cells which bind to phage but do not form plaque is due to the mutations of the membrane proteins.

## Impact of Research:

Over 80% of failed fermentation by mesophilic starter cultures are the result of bacteriophage attack. In our last report, we showed that mimic receptors i.e., rhamnose can be used to inhibit infection of phages from L. lactis subsp. cremoris KH. In this report, we demonstrated that rhamnose is also effective in inhibiting or sufficiently delaying infection of phages from L. lactis subsp. lactis C2. Elimination of bacteriophage problems during fermentation means a more

efficient cheese production, more uniform products and better consumer acceptance of the products. Without doubt, this translates into better profits to the dairy companies and farmers.

#### Publication and Abstracts:

"Characterization of Bacteriophage Receptors of lactic acid bacteria" R. Valyasevi, B. L. Geller and W. E. Sandine. American Dairy Science Association Conference, Lexington, Kentucky (July 1989).

"The Bacteriophage kh Receptor of Lactococcus lactis subsp. cremoris KH is the Rhamnose of the extracellular wall polysaccharide" R. Valyasevi, W. E. Sandine and B. L. Geller. Appl. Environ. Microbiol. (in press)

#### Patents:

Use of rhamnose and rhamnose containing materials to inhibit bacteriophages of lactic acid and related bacteria. A disclosure document has been filed with the Oregon State University Technology Transfer Office. It is being evaluated by patent attorneys.

Table 1. Phage Binding and Saccharide Analysis of Resistant Mutants

strain <sup>a</sup>	% of phage binding		carbohydrate <sup>b</sup> (ug/mg cell wall)			
	c2	sk1	Rham	Gal	Glu	Acglc
C2	98	93	228	43.4	72.5	86.0
RMSK1-1	16	36	568	33.9	38.2	113
RMSK1-2	46	63	565	39.8	55.5	117
RMSK1-3	71	79	401	32.5	48.9	89.6

<sup>a</sup> C2, wild type strain C2; RMSK1, phage resistant mutant.

<sup>b</sup> Rham, rhamnose; Gal, galactose; Glu, glucose; Acglc, N-acetylglucosamine



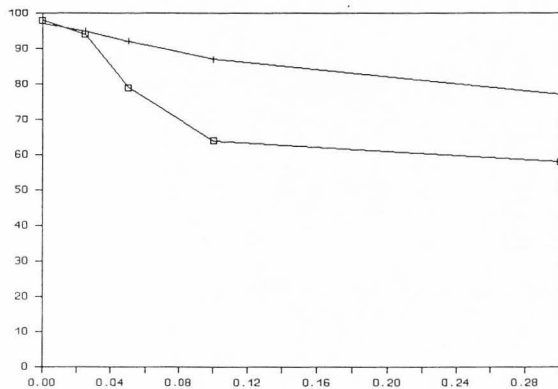


Figure 1. Effects of different concentrations of lectins from Conavalin A (□) and V. faba (+)

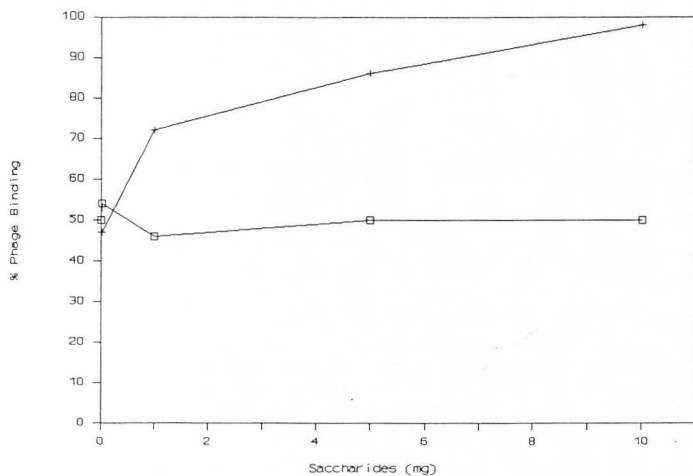


Figure 2. Glucose reverses lectin inhibition of phage sk1 binding. Symbols: glucose (+) and galactose (□).

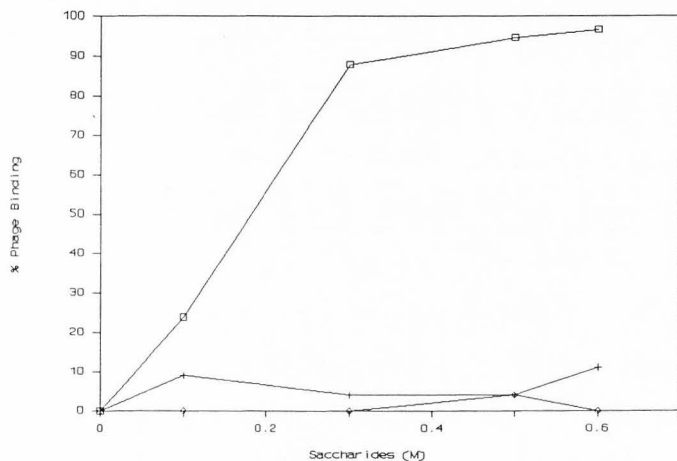


Figure 3. Effects of different concentrations of saccharides on the infectivity of phage sk1. Saccharides:  $\square$  , L(+)-rhamnose;  $\diamond$  , D(+)-glucose and  $+$  , D(+)-galactose.

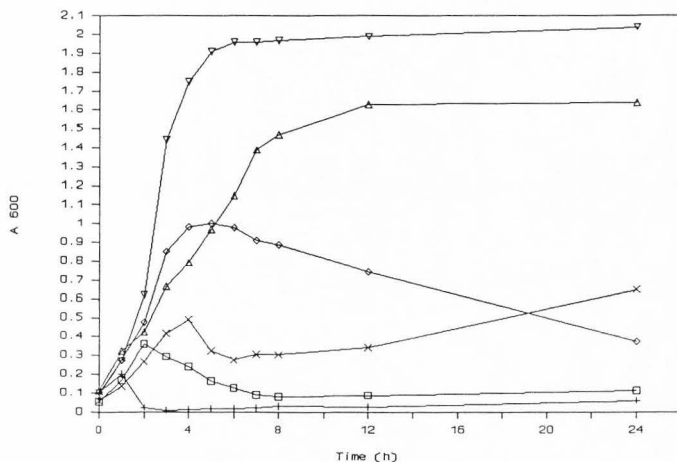


Figure 4. The effects of L(+) rhamnose on the growth of phage-infected liquid cultures of *L. lactis* subsp. *lactis* C2 and *L. lactis* subsp. *lactis* var diacetylactis 18-16. Symbols:  $\diamond$ , 0.6M rhamnose and phage sk1;  $\Delta$ , 0.6M rhamnose and phage c2;  $\square$ , phage sk1;  $+$ , phage c2;  $\times$ , phage 18-16 and *L. lactis* subsp. *lactis* var diacetylactis 18-16 and  $\nabla$ , without phage

**Project Title:** Characterization of Bacteriophage Receptor Sites of Lactococcus Bacteria

**Personnel:** B.L. Geller and W.E. Sandine, Oregon State University.

**Funding:** Western Dairy Foods Research Center

### Objectives:

The overall objective is to understand the molecular mechanism of phage adsorption to the surfaces of lactococcus bacteria. The specific objectives are:

1. To identify the cell surface location of the bacteriophage receptor.
2. To define the bacteriophage receptor at the molecular level.

### Results:

Our results can be summarized in four groups:

1. We have found that the receptor for the bacteriophage kh on *Lactococcus lactis* subsp. *cremoris* KH is the rhamnose of the extracellular wall polysaccharide. In addition, galactose is required, but appears not to directly interact with the phage. We also found that phage infection was prevented by adding rhamnose to either the phage or growing cultures.
2. The phage skl receptor on *L. lactis* subsp. *lactis* C2 was found to be the rhamnose of the exopolysaccharide. In addition, glucose appears to be required, although it is probably not the primary binding site.
3. We have tentatively identified the phage c2 receptor on *L. lactis* subsp. *lactis* C2 as the rhamnose of the exopolysaccharide. Neither galactose, glucose or N-acetylglucosamine are involved. The binding of phage c2 apparently is reversible. This is in contrast to the binding of phage skl, which is irreversible.
4. We have identified and partially purified a membrane protein required for bacteriophage c2 and skl infection of *L. lactis* subsp. *lactis* C2. The protein has an apparent  $M_r = 350,000$  and a subunit size of 32 KDa.

### Impact of Research:

Over 80% of failed fermentation by mesophilic starter cultures are the result of bacteriophage attack. In order to design rational ways of combating this very

significant economical problem of the cheese-producing industry, more must be known about the mechanisms of phage infection in lactococci. One very sensible way of eliminating this problem is to prevent the initial attachment of the phage to the starter culture cells. Without attachment, infection is impossible. Our research has begun to determine the molecular determinants of the phage receptors on the surface of different strains of *Lactococcus lactis*. Two potential approaches for preventing phage attachment have been elucidated from our results:

1. Soluble rhamnose added to the growth medium can inactivate at least three phages, or prevent infection of a growing culture. While it would be impractical to add rhamnose to the milk used in making cheese, polymers of rhamnose might be added to milk to act as a phage "adsorbent". These polymers might even be secreted by lactococci genetically programmed to synthesize such polysaccharides.
2. A membrane protein which we have found can be mutated to an extent which prevents phage infection, but has no apparent effect on the growth of the bacteria. This would suggest that an improved strain could be constructed by genetically reprogramming the starter culture with an appropriately mutated and stable copy of the required protein.

#### Abstracts of presentations at meetings:

Valyasevi, R., B.L. Geller, and W.E. Sandine. 1989. Characterization of bacteriophage receptor sites of *Lactococcus* bacteria. Oregon Dairy Industries Conference, February 15-16, 1989, Eugene Oregon.

Valyasevi, R., B.L. Geller, and W.E. Sandine. 1989. Characterization of bacteriophage receptor sites for lactococci. Presented at the American Dairy Science Association annual conference, Lexington, Kentucky, July 31- August 4. J. Dairy Sci. Suppl. 1, 145.

Sandine, W.E. and B.L. Geller. 1989. Characterization of bacteriophage receptor sites of *Lactococcus* bacteria. Western Dairy Foods Research Center Annual Meeting. July 13-14, 1989, Logan, Utah.

Valyasevi, R., W.E. Sandine and B.L. Geller. 1990. A new approach to control bacteriophage for lactic acid bacteria. Oregon Dairy Industries Conference, February 14, 1990, Eugene Oregon.

Geller, B.L., W.E. Sandine, and R. Valyasevi. 1990. Characterization of bacteriophage receptor sites of *Lactococcus* bacteria. Western Dairy foods Research Center Annual Meeting. July 12-13, 1990, Corvallis, Oregon.

Geller, B.L., W.E. Sandine, R. Valyasevi. 1990. Characterization of bacteriophage receptors of *Lactococcus* bacteria. Western Dairy Foods Research Center national meeting. August 22-23, Logan, Utah.

ler, B.L., W.E. Sandine, and R. Valyasevi. 1991. A 32 KDa membrane protein of *Stococcus lactic* subsp. *lactis* C2 is required for phage infection. Submitted to The American Dairy Science Association annual meeting. August 12-15, Logan, Utah.

#### Applications:

Valyasevi, R., W.E. Sandine, and B.L. Geller. 1990. Bacteriophage KH receptor of *Stococcus lactic* subsp. *cremoris* KH is the rhamnose of the extracellular wall polysaccharide. Appl. Environ. Microbiol. 56:1882-1892.

Valyasevi, R., W.E. Sandine, and B.L. Geller. 1991. A membrane protein of *Stococcus lactic* subsp. *lactis* C2 is required for phage infection. J. Bacteriol., in press.

#### Conclusions:

Valyasevi, R., W.E. Sandine, and B.L. Geller. Bacteriophage skl receptor is the rhamnose and glucose of the extracellular wall polysaccharide of *Lactococcus lactis* subsp. *lactis* C2. Submitted.

Valyasevi, R., W.E. Sandine, and B.L. Geller. Bacteriophage c2 receptor of *Stococcus lactic* subsp. *lactis* C2 is the rhamnose of the extracellular wall polysaccharide. In preparation.

**Project Title:** Purification of a bacteriocin from *Pediococcus pentosaceus* and genetic transfer of the plasmid borne determinant

**Personnel:** Mark A. Daeschel, Assistant Professor, Food Science and Technology, Oregon State University

Ms. Xintian Ming, Graduate Student, Food Science and Technology, Oregon State University.

**Funding Sources:** Western Dairy Foods Research Center  
Oregon Agricultural Experiment Station.

### Objectives:

The primary objectives of this study are twofold.

1. To purify the bacteriocin, Pediocin A, using protein purification methodology to a purity suitable for the production of polyclonal antibodies.
2. To genetically transfer the pediocin A plasmid (pMD136) into dairy fermentation strains via the current state of the genetic transfer systems that have been demonstrated with lactic acid bacteria.

### Results:

This project has just finished its first year of work during which time we have focused on concentration and purification of Pediocin A to the extent where it could be incorporated into a selective medium suitable for the recovery of transformants. We have not been able to demonstrate activity in culture supernatants in which Pediocin A producing *Pediococcus pentosaceus* has been cultivated. Ultrafiltration or dialysis concentration of supernatants did not result in detection of Pediocin A activity when assayed by the well diffusion method. However, activity is clearly seen with the deferred antagonism method using the same medium (Trypticase Soy Broth).

A concurrent approach to developing a selection system for recovering Pediocin A transformants is to utilize the antagonistic effect that Pediocin A transformants would have on untransformed recipients. Previous data (Daeschel, unpublished) has shown the frequency of (Bac<sup>-</sup>) cells within population harboring pMD136 ranged from .001% to 99.9% depending on temperature of cultivation. Populations that were predominantly (Bac<sup>-</sup>) after cultivation at 42 C became predominantly (Bac<sup>+</sup>) after several growth transfers at 37 C. This may reflect the "self selecting" of the bacteriocin-immunity genotype where plasmid (pMD136) cured cells may be vulnerable to the bacteriocin. Our next experimental approach will be to utilize these observations to develop a selection system for Pediocin A transformants based on their ability to predominate over untransformed cells after several growth cycles.



### Economic Impact:

Modern genetics has provided the tools for construction lactic acid bacteria with the ability to enhance the quality of fermented dairy foods and to provide a higher degree of preservation. Genetic transfer systems such as transformation, transduction and conjugation have been demonstrated in lactic acid bacteria and are currently being used by research in dairy starter cultures for strain improvement. These technologies will allow us to transfer into dairy cultures the genes that encode for antagonistic substances (bacteriocins) that are active against a variety of undesirable spoilage and pathogenic microorganisms that occur in fermented dairy foods. The inhibition of undesirable microorganisms will allow for enhanced product quality and safety which in the long run will enhance milk utilization.

Of the current concern to cheese processors are the occurrence of microorganisms which cause bloating faults (*Clostridium tyrobutyricum*) in Swiss-type cheese and *Listeria monocytogenes* a pathogen which can contaminate milk and soft cheeses. Bacteriocin producing starter cultures is one approach for controlling the incidence of such microorganisms in fermented dairy foods. Certain bacteriocins from non-dairy lactic acid bacteria have been shown to inhibit clostridia and *Listeria* as well as other pathogens such as *Staphylococcus aureus*. The acquisition of bacteriocin producing ability by dairy starter cultures through genetic biotechnology may allow the development of strains superior to those presently available.

**Project Title:** Purification of a bacteriocin from Pediococcus pentosaceus and genetic transfer of the plasmid borne determinants

**Personnel:** Mark A. Daeschel, Principal Investigator, Dept. of Food Science and Technology, Oregon State University

Xintian Ming, Dept. of Food Science and Technology, Oregon State University

**Funding Sources:** Western Dairy Foods Research Center

### **Objectives:**

The primary objectives of this study are twofold:

To purify the bacteriocin, Pediocin A, using protein purification methodology to a purity suitable for the production of polyclonal antibodies.

To genetically transfer the Pediocin A plasmid (pMD136) into dairy fermentation strains via the current state of the genetic transfer systems that have been demonstrated with lactic acid bacteria.

### **Results:**

Initial studies were focused on concentration and purification of Pediocin A to the extent where it could be incorporated into a selective medium suitable for the recovery of transformants. We have not been able to demonstrate activity in culture supernatants in which Pediocin A producing Pediococcus pentosaceus has been cultivated. Ultrafiltration and dialysis concentration of supernatants did not result in detection of Pediocin A activity when assayed by the well diffusion method. However, activity is clearly seen with the agar spot antagonism method using the same medium (Trypticase Soy Broth).

During the 2nd year we concentrated on optimizing electroporation transformation procedures for Pediococci and Lactococci with the view of introducing the Pediocin A plasmid since we are unable at this time to use Pediocin A as a selective agent. Two alternative approaches are being explored. The first approach is to optimize electroporation conditions so that co-transformation of pMD136 and a directly selectable marker (antibiotic resistant plasmid) could occur. Co-transformants could then be individually screened for Pediocin A production. The second approach is to clone into a directly selectable plasmid vector the Pediocin A genes for production and immunity to Pediocin A. Both approaches are dependent on high transformation frequencies. We have been able to transform 3 of 5 strains of pediococci using the plasmid vector pNZ12. Transformation frequencies were in the range of  $2-3 \times 10^2$  per  $\mu\text{g}$  of DNA. We have also been able to obtain transformation frequencies of about  $1 \times 10^6$  per  $\mu\text{g}$  of DNA with Lactococcus lactis LM 2302 which will be used as a recipient for pMD136- pNZ12 co-

transformation experiments.

During the past year we have redoubled our efforts toward isolating active Pediocin A. Various media formulations were evaluated in both deferred antagonism and supernatant assays. We still have been unable to make significant progress in isolating enough active pediocin A in sufficient quantities for use in plasmid transformation experiments. It appears that Pediocin A may be a very fragile protein in terms of maintaining bioactivity. We believe that physical manipulations may accelerate loss of activity. During the remainder of the project period (till January 1, 1992) we will explore other isolation and concentration procedures that minimize physical stresses to proteins.

### Impact of Research:

Modern genetics has provided the tools for constructing lactic acid bacteria with the ability to enhance the quality of fermented dairy foods and to provide a higher degree of preservation. Genetic transfer systems such as transformation, transduction and conjugation have been demonstrated in lactic acid bacteria and are currently being used by researchers in dairy starter cultures for strain improvement. These technologies will allow us to transfer into dairy cultures the genes that encode for antagonistic substances (bacteriocins) that are active against a variety of undesirable spoilage and pathogenic microorganisms that occur in fermented dairy foods. The inhibition of undesirable microorganisms will allow for enhanced product quality and safety which in the long run will enhance milk utilization.

One of current concern to cheese processors are the occurrence of microorganisms which cause blowing faults (Clostridia tyrobutyricum) in Swiss-type cheese and cheeses. Bacteriocin producing starter cultures is one approach for controlling the incidence of such microorganisms in fermented dairy foods. Certain bacteriocins from non-dairy lactic acid bacteria have been shown to inhibit clostridia and Listeria as well as other pathogens such as Staphylococcus aureus.

The acquisition of bacteriocin producing ability by dairy starter cultures through genetic biotechnology may allow the development of strains superior to those presently available.

### Publication and Abstracts:

alone

### Patents:

alone

**Project Title:** Prediction and Determination of the Efficacy of Nisin in Dairy Foods

**Personnel:** M.A. Daeschel, Assistant Professor, Dept. of Food Science and Technology, Oregon State University

F.W. Bodyfelt, Professor, Dept. of Food Science and Technology, Oregon State University

Nancy Eid, Student and Student Worker, Dept. of Food Science and Technology, Oregon State University

Randy Bender, Research Assistant, Dept. of Food Science and Technology, Oregon State University

**Funding Sources:** Western Dairy Foods Research Center  
Oregon Agricultural Experiment Station

### Objectives:

1. To determine what molecular components of milk can interact with nisin and affect its activity.
2. To use the information gained from achieving the first objective to predict and determine the efficacy of nisin as a preservation agent in novel dairy foods such as carbonated milk beverages.
3. To determine the efficacy of nisin on the inhibition of *Bacillus* sp. in selected milk and milk products and the subsequent impact on keeping quality.

### Results:

Experiments were conducted to determine what specific components of dairy foods could enhance or diminish the activity of nisin. Nisin has been reported to be adsorbed to protein components of certain foods. We examined a series of proteins including the milk protein, Beta-lactoglobulin, for their effect on nisin activity in aqueous solution. Fig. 1 (c) shows a protective effect of Beta-lactoglobulin on nisin sensitive bacteria when exposed to nisin. This is evidenced by the growth of the seeded bacteria in the area where Beta-lactoglobulin has diffused from the well into the medium. The outer ring of inhibition in well C is due to nisin diffusing faster than Beta-lactoglobulin.

Experiments were also conducted to test the effect of milk fat on the activity of nisin. Nisin was added to non-fat milk, 4% milk and heavy cream and then dialyzed against pH 6.5 buffer in membrane bags and then assayed for activity at 24 hour intervals. The activity in the dialysates was greatest with non-fat milk and the least with heavy cream. This suggests that milk fat can reduce nisin activity by a binding type of mechanism.

### Impact of Research:

Nisin is a member of a group of potent antibacterial substances which are bacteriocins. It has been shown to be effective in inhibiting certain gram positive species but not gram negative bacteria, yeasts or fungi. Recent investigations have indicated that nisin and/or nisin producing streptococci are inhibitory toward *Listeria monocytogenes*, a foodborne pathogen of emerging concern.

Nisin, after 25 years of safe use in many European countries was recently affirmed by the Food and Drug Administration (Federal Register, April 6, 1988) as GRAS for use as an antimicrobial agent to inhibit the outgrowth of *Clostridium botulinum* spores and toxin formation in certain pasteurized cheese spreads. The approval of nisin will justify increased research efforts of both an applied and basic nature on the antimicrobial properties of bacteriocins. It is this author's opinion that nisin will eventually be approved for use in other types of dairy foods once a sufficient body of scientific knowledge has been accumulated to justify approval. The use of nisin as an antimicrobial agent in dairy foods could enhance milk utilization by at least three mechanisms:

- 1 ) Inhibition of spoilage microorganisms in dairy foods could minimize economic losses due to spoilage.
- 2) Inhibition of pathogenic and toxigenic bacteria to provide consistently safe products. Contaminated products (such as with *Listeria*) can give rise to adverse publicity with subsequent sales loss.
- 3 ) Extension of the shelf-life of perishable dairy products .

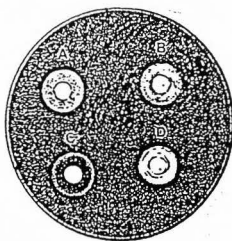


Figure 1. Effect of Beta-lactoglobulin of the ability of nisin to inhibit a nisin sensitive bacterium in a seeded agar assay

- A.) 0.1 mg/ml Beta-lactoglobulin + 100 nisin/ml
- B.) 1.0 mg/ml Beta-lactoglobulin + 100 nisin/ml
- C.) 10 mg/ml Beta-lactoglobulin + 100 nisin/ml
- D.) 100 U nisin / ml

**Project Title:** Prediction and Determination of the Efficacy of Nisin in Dairy Foods

**Personnel:** Mark A. Daeschel, Principal Investigator, Dept. of Food Science and Technology, Oregon State University

Floyd W. Bodyfelt, Principal Investigator, Dept. of Food Science and Technology, Oregon State University

Dong-Sun Jung, Graduate Research Assistant, Dept. of Food Science and Technology, Oregon State University

**Funding Sources:** Western Dairy Foods Research Center

**Objectives:**

To determine what molecular components of milk can interact with nisin and affect its activity.

To use the information gained from achieving the first objective to predict and determine the efficacy of nisin as a preservation agent in novel dairy foods such as carbonated milk beverages.

To determine the efficacy of nisin on the inhibition of Listeria and psychrotrophic bacilli in selected milk and milk products and the subsequent impact on safety and keeping quality.

Documentation of how food grade emulsifiers enhance nisin activity against Listeria monocytogenes in high fat containing fluid milk.

**Results:**

Our previous report provided information on the effect of dairy proteins on nisin activity.  $\kappa$ -lactoglobulin was observed to provide a protective effect to bioassay indicator bacteria when exposed to nisin in the presence of the protein. It is hypothesized that  $\kappa$ -lactoglobulin may reduce the activity of nisin by binding it and hence preventing it from inhibiting microbial cells.

More experiments were conducted to determine the effects that various components of milk have on the efficacy of nisin. Two approaches were used. 1) Determination of nisin activity after exposure of nisin to different types and concentration of dairy proteins and fats. A quantitative bioassay based on well diffusion was employed. 2) Effect of different dairy components on the ability of nisin to inhibit Listeria monocytogenes in fluid milk. The most significant effect observed was the reduction in nisin activity as the fat concentration was increased in fluid milk. Nisin activity as determined by bioassay was decreased by

more than 90% when added to high fat (11.5%) fluid milk. Concurrently, it was observed that nisin was less effective in inhibiting *Listeria* as fat concentration increased. A representative experiment is portrayed in the following figure. (Fig. 1)

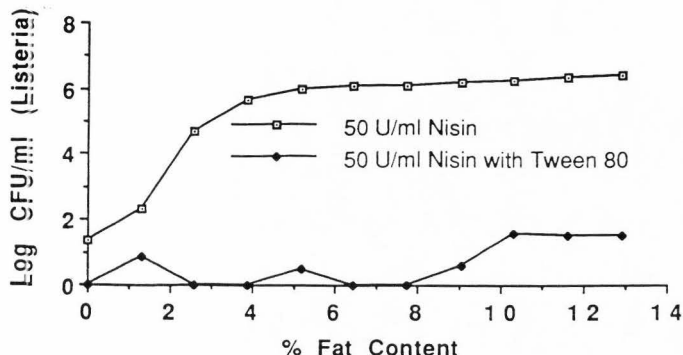


Figure 1. Effect of % milkfat content of fluid milk on the ability of nisin to inhibit *L. monocytogenes*. Sterile milks containing 0, 10, and 50 U/ml nisin were inoculated with *L. monocytogenes* Jalisco at levels [log<sub>10</sub> 7.15 cfu/ml for 0 U/ml, 7.57 for 1 U/ml and 7.25 for 50 U/ml].

The newly identified project objective; "Documentation of how food grade emulsifiers enhance nisin activity against *Listeria monocytogenes* in high fat containing fluid milk" was formed on our experimental observation that the emulsifier Tween 80 appeared to "restore" nisin activity in milks containing high amounts of milkfat (4-13%) Fig. 2 and Table 1. Our working hypothesis to explain this observation is: We believe that nisin can bind to the surface of milkfat globules and when this occurs, nisin is no longer active or available to inhibit bacteria. The higher the fat content of milk, the less active or available to inhibit bacteria. The higher the fat content of milk, the less active nisin is. We believe the addition of the emulsifier Tween 80 to milk acts to displace proteins (nisin) from the milkfat globule surface resulting in a restoration of nisin activity.

TABLE 1. Effect of emulsifiers on nisin activity in half-and-half cream and skim milk

<u>Sample treatment</u> (all contain 50 U/ml nisin)	<u>Inhibition zone dia. (mm)</u>	<u>% nisin activity</u>
skim milk	24.0	82.7
skim milk+Tween 80	24.8	100
skim milk+lecithin	23.8	78.9
half+half cream (h+h)	16.2	19.6
(h+h)+Tween 80	21.0	43.4
(h+h)+ lecithin	16.0	19.1
nisin 50 U/ml	24.8	100
(controls) do not contain nisin		
skim milk	0	n/a
(h+h)	0	n/a
Tween 80	0	n/a
lecithin	0	n/a

n/a=not applicable

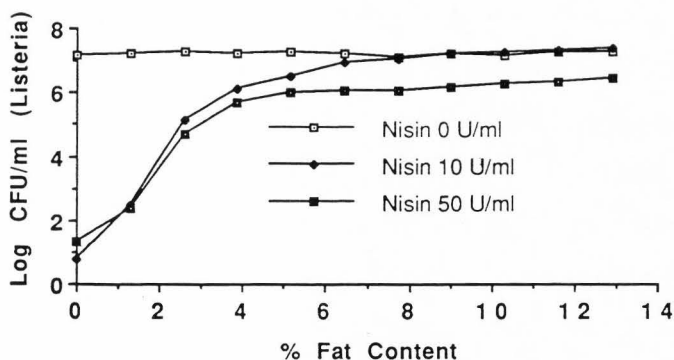


Figure 2. Effect of Tween 80 (0.2% vol/vol) on the efficacy of nisin (50 U/ml) in milks with varying fat content. Sterile milks were inoculated with *L. monocytogenes* Jalisco at levels  $10^{7.20}$  cfu/ml for treatments without Tween 80 and  $10^{7.60}$  cfu/ml with Tween 80].



The importance or implications of these observations to the dairy industry are both relevant and practical. Nisin has been approved as a GRAS additive in cheese spreads and will likely be approved for use in other dairy products. An understanding of how nisin interacts with dairy food components is paramount in order to optimize its efficacy and application in dairy foods.

We plan to experimentally prove (or disprove) our hypothesis by documenting the adsorption or binding to the milkfat globule and its subsequent desorption. This will be accomplished by labeling the nisin molecule with a fluorescent probe and microscopically visualizing its adsorption to milkfat globules. Desorption by Tween 80 will be documented in the same manner. This information will corroborate our existing data on nisin activity measurements as affected by milkfat and Tween 80.

### **Impact of Research:**

Nisin is a member of a group of potent antibacterial substances which are bacteriocins. It has been shown to be effective in inhibiting certain gram positive species but not gram negative bacteria, yeasts or fungi. Recent investigations have indicated that nisin and/or nisin producing streptococci are inhibitory toward Listeria monocytogenes, a food-borne pathogen of emerging concern.

Nisin, after 25 years of safe use in many European countries was recently affirmed by the Food and Drug Administration (Federal Register, April 6, 1988) as GRAS for use as an antimicrobial agent to inhibit the outgrowth of Clostridium botulinum spores and toxin formation in certain pasteurized cheese spreads. The approval of nisin will justify increased research efforts of both an applied and basic nature on the antimicrobial properties of bacteriocins. It is this author's opinion that nisin will eventually be approved for use in other types of dairy foods once a sufficient body of scientific knowledge has been accumulated to justify approval. The use of nisin as an antimicrobial agent in dairy foods could enhance milk utilization by at least three mechanisms:

1. Inhibition of spoilage microorganisms in dairy foods could minimize economic losses due to spoilage.
2. Inhibition of pathogenic and toxigenic bacteria to provide consistently safe products. Contaminated products (such as with *Listeria*) can give rise to adverse publicity with subsequent sales loss.
3. Extension of the shelf-life of perishable dairy products.

**blications:**

- Daescheel, M. A., D. S. Jung and F. W. Bodyfelt. 1990. Influence of fat on the efficacy of nisin in inhibiting Listeria monocytogenes in fluid milk. Abstract in J. Dairy Sci. 73(6):Suppl. 1.
- Jung, D. S., F. W. Bodyfelt and M. A. Daeschel. 1991. Influence of fat and emulsifiers on the efficacy of nisin in inhibiting Listeria monocytogenes in fluid milk. Submitted, J. Dairy Science 5/30/91.

**Project Title:** Studies on growth and survival of *Bifidobacterium* species in milk.

**Personnel:** J. W. Ayres, Professor, School of Pharmacy, Oregon State University

W. E. Sandine, Professor, Department of Microbiology, Oregon State University

Ronshan Cheng, Graduate Student, Department of Microbiology, Oregon State University.

**Funding Sources:** Western Dairy Foods Research Center  
Oregon Agricultural Experiment Station

### Objectives:

1. To study the requirements of bifidobacteria species for growth and stability in milk.
2. To use the oxygen consuming membrane fraction (oxyrase) of *E. coli* to provide anaerobiosis during the growth of bifidobacteria in milk.
3. To use oxyrase in improving plating condition for enumeration of bifidobacteria in various products.
4. To use newly acquired customized state of the art fermentation equipment to optimize growth parameters to maximize cell numbers and stability.
5. To determine the best delivery system for bifidobacteria cells (lyophilized or frozen concentrates) to produce a sweet bifidus milk.
6. To study the associative growth of bifidobacterial species with other organisms used in dairy fermentations such as lactobacilli, lactococci, and *Leuconostoc*.

### Results:

A whey-based medium (7% sweet whey, 0.05% cysteine and 0.3% yeast extract, WCY-0.3) was found satisfactory for growth of a variety of *Bifidobacterium* species without using anaerobic conditions. Freshly pasteurized (90°C, 45 min) and cooled (37°C) WCY-0.3 was inoculated with  $0.2\%$  ( $10^6$  to  $10^7$  CFU/ml) of the following active *Bifidobacterium*: *B. bifidum* 15696, *B. breve* 15700, *B. longum* 15707, *B. breve* 15698, *B. longum* L10, *B. longum* L12, and *B. longum* 3J. Following incubation for 12 hr, most strains reached maximum cell densities of  $10^9$  to  $5 \times 10^9$  CFU/ml, except *B. bifidum* 15696 and *B. longum* 3J. Addition of the oxyrase to the WCY (7% sweet whey and 0.05% cysteine with any level of yeast extract) at 0.03 units/ml (WCOY) reduced the lag phase of all strains, allowing maximum populations to be achieved more quickly. Boiled oxyrase (0.3 unit/ml, 100°C for 10 min) was found to be a growth stimulant for strain 15707, but not for the others. Two to seven times of population densities could be reached in the WCOY-0.3 medium of four tested strains 15696, 15700, 15707, L10 by incorporating of 1.9% disodium glycerophosphate or 1.9% trimagnesium phosphate before 12 h incubation at 37°C. The viability of these strains was retained throughout a 24-hr incubation period, in contrast to rapid death of

**Project Title:****Generation of Molecular Probes for  
Bifidobacterial Species****Personnel:**

Joseph W. Booth, Dept. of Biochemistry and  
Biophysics, Oregon State University

Janine E. Trempey, Dept. of Microbiology, OSU

William E. Sandine, Dept. of Microbiology, OSU

**Funding:**

Western Dairy Foods Research Center

**Objectives:**

The popularity of products containing viable bifidobacterial cultures is well known in Europe and Japan and has risen to a certain extent in recent years in such products as yogurt in the U. S. as well. The difficulty with using bifidobacteria as adjuncts in dairy products has been their instability in culture with other lactic acid bacteria. Research into culturing protocols which enable the survival of bifidobacteria when grown with other lactic acid bacteria is in great need but is attenuated by difficulty in quantitating bifidobacteria in culture with other organisms. Attempts to develop a selective plating medium to aid in quantitating bifidobacteria in culture with other organisms have been only marginally successful. We have proposed utilization of modern molecular biology to aid in the difficult problem of quantitating bifidobacteria in culture. The present report updates research into making molecular probes which will be used to tag and identify individual bifidobacterial colonies on colony lifts.

The molecular probes whose generation is sought are (1) an antibody probe against fructose-6-phosphate phosphoketolase--anti-F6PPK, and (2) a DNA probe with a nucleic acid sequence complementary to a portion of the F6PPK gene. The anti-F6PPK probe will quantitate bifidobacterial species by Western blotting colony lifts of plated cultures. The DNA probe will quantitate bifidobacterial species by Southern blots of colony lifts. The F6PPK gene product was chosen as a tag for identification of bifidobacteria because it participates in a unique carbohydrate metabolism pathway known to exist only in bifidobacteria. To generate an antibody probe it is necessary to first purify the F6PPK enzyme. To generate a DNA probe it will be necessary to clone and sequence the gene for F6PPK.

1. Purify the F6PPK enzyme and obtain antisera against the enzyme to use for Western blots.

2.. Construct a library of genomic sequences of a Bifidobacterium specie; identify a clone which contains the gene for F6PPK using a probe deduced from the peptide sequence of the F6PPK protein; sequence the gene for F6PPK; use the sequence of F6PPK to construct DNA probes which can be used in Southern blots to identify bifidobacterial colonies.

### **Results:**

Significant protein purification of the F6PPK enzyme has been achieved using protamine sulfate to fractionate cell-free bifidobacterial extracts. We have found that a 1.474 mg/ml final concentration of protamine sulfate precipitates nucleic acid and unwanted proteins while a concentration of 1.534 mg/ml final concentration of protamine sulfate quantitatively precipitates the F6PPK enzyme.

Used in conjunction with protamine sulfate precipitation, native gel electrophoresis is capable of producing a single band on a gel with F6PPK activity. Native gel electrophoresis will be useful in purifying the F6PPK enzyme.

Experiments are currently underway to generate a library of Bifidobacterium breve genomic sequences.

### **Impact of Research:**

(Generation of molecular probes for the identification of bifidobacterial species will make possible the accurate determination of numbers of viable bifidobacterial cells in mixed cultures containing bifidobacteria and other lactic acid bacteria. This will facilitate making a variety of bifidobacterial-containing milk products of uniform quality where numbers of these bacteria are concerned.

### **Publications:**

None

**Project Title:** Characterization of Milk Proteolysis by Lactococcal Starter Culture Strains Using Amino Acid Analysis

**Personnel:** Rodney J. Brown, Dept. of Nutrition and Food Sciences, Utah State University

Christina Beer, Dept. of Nutrition and Food Sciences, Utah State University

**Funding:** Western Dairy Foods Research Center

**Objectives:**

Use amino acid analysis to study the interactions of the proteinase genes in *Lactococcus lactis* ssp. *lactis* cultures that exhibit different proteinase phenotypes.

Stabilize the proteinase gene system in the organism.

Find the optimum expression of proteinase genes to improve flavor and texture characteristics in cheese, and to be used for accelerated ripening.

**Results:**

A new graduate student has begun work on this project. She is becoming familiar with the o-phthaldialdehyde test and amino acid analysis used to characterize proteolysis of milk proteins during growth, the patterns of individual amino acid concentrations used to characterize proteolysis, and cluster analysis for differentiating strains beyond what was possible by visual comparison of the amino acid analysis results. In addition, she is working with the proteinase gene system of *Lactococcus lactis* ssp. *lactis* cultures.

**Impact of Research:**

Proteolysis from the bacterial starter cultures plays a significant role in the physical and organoleptic properties of cheese and other fermented dairy products. Improper proteolysis can result in a wide number of defects, including bitterness, texture, and flavor problems. We have ways to measure gross proteolysis but are very limited in techniques to profile or characterize proteolysis for individual bacterial strains. The proteinase system in Lactococci is very complex and phenotypic changes relative to genetic manipulation of a culture are impossible to measure with present proteolysis tests. This method would allow us to measure subtle differences in proteolysis as the genetic complement of a culture is modified. An understanding of the interactions of the various genetic factors would allow for the development and optimizations of cultures used in dairy fermentations. This project has the potential to greatly enhance product quality, allow for the production of products with enhanced properties, and even allow development of new products by using bacterial strains with different proteolytic abilities. This method would also be very valuable in identifying and characterizing newly developed strains from biotechnological endeavors.